Elucidating the role of cation/proton exchangers (CAX) in maintaining ion homeostasis and modulating Ca\(^{2+}\) signalling in *Chlamydomonas reinhardtii*  

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy  
In the Faculty of Life Sciences  

2014  

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Abstract:

Calcium (Ca\(^{2+}\)) is a ubiquitous ion with signalling function that forms part of a plethora of signal transduction pathways which allow plants to respond to environmental stimuli in a stress-specific manner. Although we know a certain amount about the shape of Ca\(^{2+}\) signals, and the downstream processes regulated by Ca\(^{2+}\) signalling, the role of specific Ca\(^{2+}\) transporters in modulating these signals is yet to be fully understood. Plant Ca\(^{2+}/H^+\) exchangers (CAX) are of strong interest to study in terms of Ca\(^{2+}\) signalling due to their high capacity sequestration of Ca\(^{2+}\), as well as their localisation at the vacuolar membrane, with the vacuole being a major Ca\(^{2+}\) store in plant cells. Due to the high gene redundancy observed in plants, however, it has been difficult to gauge their precise role. In order to better understand the role that CAX transporters play, the unicellular green alga *Chlamydomonas reinhardtii* was used as a model to explore Ca\(^{2+}\) signalling mechanisms in higher plants, in particular the role of CAX in maintaining ion homeostasis and modulating Ca\(^{2+}\) signals. The data presented here shows, for the first time, that *C. reinhardtii* cells respond to environmental stimuli such as NaCl and CdCl\(_2\) with rapid, transient Ca\(^{2+}\) elevations. The NaCl-induced Ca\(^{2+}\) elevations were also found to increase in magnitude with increased stimulus. Analysis of *CAX1* and *CAX2* knockdowns of *C. reinhardtii* suggest that CAX function is necessary to maintain a balance of nutrients including Ca, Cu, Fe, K, S, Mg, Mn and Zn. *CAX1* and *CAX2* knockdowns showed opposing phenotypes in response to ion stress, with *CAX1* knockdowns showing reduced growth on CaCl\(_2\) and NaCl but *CAX2* knockdowns showing increased growth. Initial Ca\(^{2+}\) signalling experiments suggest also that CAX1 of *C. reinhardtii* is important in maintaining an intracellular store of Ca\(^{2+}\) prior to NaCl-induced Ca\(^{2+}\) release.
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Acknowledgements:

I have had a wonderful four years journeying between Manchester and Plymouth and I am extremely grateful for all of the opportunities this PhD had provided. First and foremost, I would like to thank Jon Pittman and Glen Wheeler for their excellent supervision, training, guidance and patience during the course of my PhD, and to Simon Turner and Colin Brownlee for their advice. Thanks also to the BBSRC who provided me with funding throughout, as well as to the MBA who hosted me for eight months over two years and the University of Manchester, where I’ve been for seven. I’d also very much like to thank Rachel Webster, Andrew Dean, Paul Carr, Peter Etchells and Yaomin Cai for their help with my many, varied questions in the lab.

I would also like to thank Amanda Bamford, Rachel Webster and Andrew Dean for all the public engagement activities which they inspired me to take part in, and to Kate Woodburn who ran my first ever public engagement activity when I was still an undergraduate student. I’d also like to thank Matthew Miller, who I’ve been leaping around with in demonstrations and public engagement events for four years (and around Ecuador, Manchester, and elsewhere for seven), which has helped me to be continuously inspired and enthusiastic about science, as well as Nick Zoulias, who is always about for an amazing chat, advice, and tonnes of coffee. I’d like also to thank the various people who have made this PhD experience so wonderful, including my lab-mates (past and present) Adam, Amit, Anifon, Mayo, Tom, Ben, Guillaume and Helena as well as Jack, Gideon, Jen, Chris, and everybody else at the University of Manchester and the MBA who I have been fortunate enough to meet and spend some great times with. I would also like to thank Keith White, who asked me to join him on the Greece Field Trip, which was a tremendous experience, as were the many pints each Friday evening.

I would also like to thank Liz Sheffield for all of the educational opportunities which have come my way since commencing my PhD. After she had put me in contact with a school about teaching their GCSE pupils some plant science, I since visited that school
and several more around Manchester for a period of three years, and have now given talks and presentations around the country. She also was the person who inspired my interest in plant sciences in the first instance, and her Biodiversity course persuaded me to take up my interest in plants. I must also thank Liz, and Ruth Grady, for asking me to deliver lectures to undergraduate students on Biodiversity when Amanda was away on sick leave. Liz was also the person who sent me the e-mail link to take part in Thought for Food, which has become one of my major passions.

I would also like to take this opportunity to thank all of my wonderful friends and family, without who life would be pretty dull! To my amazing parents, Bernie and Dave, who have always supported me with love and kindness, and to my equally amazing sister, Heather, who is a constant source of inspiration, I am immensely grateful. Finally, I’d like to thank my wonderful girlfriend Marina, who has really helped inspire me toward completing my PhD (even teaching me how to analyse my qPCR) and who has also been immensely patient during my several months of whinging while writing this Thesis and finding a job! Grazie mille la mia pandina, mi hai reso felicissimo.

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For Allan, Nancy, Willison, Joe, Mary, Ann-Marie, Kieran, Imogen, Michael, Kath, and Graham.
Chapter 1 – Introduction:

This introduction chapter contains two figures and one paragraph of text from a review entitled, “Calcium signalling in plants” (Bickerton and Pittman 2012), which was written by me and edited by Jon Pittman. Several sections of this introduction will also be published in a review later this year, entitled, “The role of CAX in abiotic stress signalling in plants” (Bickerton and Pittman, unpublished), which was written by me and edited by Jon Pittman.
Introduction:
Calcium (Ca^{2+}) is "the fifth most abundant element in the earth’s crust" and is responsible for a variety of cell processes throughout all domains of life (Case et al. 2007). Ca^{2+} is present in the bones of vertebrates (composed mainly of calcium phosphate), as well as the calcium carbonate shells of corals and coccolithophores. Additionally, in plants Ca^{2+} is present in high amounts in the cell wall pectin matrix and confers stability in cell walls through binding pectin chains. Since the formation of the first living cell organisms have had to regulate Ca^{2+} due to its affinity for, and interaction with, cellular components (Case et al. 2007). Furthermore, it has always been important for organisms to maintain a resting cytosolic Ca^{2+} concentration ([Ca^{2+}]_{cyt}) at around 100nM because of the toxicity of Ca^{2+} to cells at high levels (Case et al. 2007). This strict regulation of [Ca^{2+}]_{cyt} has rendered Ca^{2+} a versatile secondary messenger during signal transduction of different environmental cues. Therefore, Ca^{2+} is a ubiquitous signalling molecule in Prokaryotes and Eukaryotes (Dominguez 2004, Case et al. 2007), and differential modulation of [Ca^{2+}]_{cyt} leads to a vast array of specific responses to a variety of biotic and abiotic stimuli. Considering that Ca^{2+} is such a ubiquitous and essential element to life on earth, it is very important to study the processes shaping Ca^{2+} signalling and maintaining Ca^{2+} homeostasis. Many of the mechanisms governing animal Ca^{2+} signalling have been well established (Berridge et al. 2000). However, in plants, despite an increasing knowledge of how certain stimuli can lead to a Ca^{2+} signalling response, how these signals are generated by a plethora of Ca^{2+} permeable channels (McAins and Pittman, 2009, Dodd et al. 2010), and how these can lead to stress-specific gene expression (e.g. Whalley et al. 2011), many of the processes that control the shaping and modulation of Ca^{2+} signals have yet to be properly elucidated.
Calcium signalling in plants:


Increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) are transient and take the form of brief spikes (e.g. Baum et al. 1999), extended elevations (e.g. Gong et al. 1997), oscillations (e.g. Allen et al. 2000) and waves through cells and tissues (e.g. Tucker et al. 2005, Choi et al. 2014). Another trait in plant Ca\(^{2+}\) signalling is that cells may produce a Ca\(^{2+}\) signal with a proportional magnitude to the applied stimulus so that cells do not produce an all-or-nothing response but can balance the effect of different stimuli on cellular processes (McAinsh and Hetherington 1998). McAinsh et al. (1992) have coined the phrase "calcium signature" to describe each distinctive Ca\(^{2+}\) response to a specific stimulus. It is important that plants can distinguish between different Ca\(^{2+}\) signals because many cellular processes, such as the changing of stomatal aperture, require a response to various stimuli that utilise Ca\(^{2+}\) as a secondary messenger but can often have antagonistic effects (McAinsh and Hetherington 1998). The spatiotemporal dynamics of Ca\(^{2+}\) signalling are therefore tightly regulated by a plethora of transporters which both bring Ca\(^{2+}\) into, and extrude Ca\(^{2+}\) from, the cytosol (McAinsh and Pittman 2009, Dodd et al. 2010).
Stimuli such as pathogen elicitors, ABA, H₂O₂ and cold, show specific cytosolic Ca²⁺ signatures after influx of Ca²⁺. Ca²⁺ binds to sensors such as calmodulin (CaM), CaM-like proteins (CML), calcium-dependent protein kinases (CDPK), and calcineurin B-like proteins (CBL) (which forms a complex with Ca²⁺-independent protein kinase (CIPK)). Ca²⁺ sensors activate transcription factors (TF) and other proteins either by direct binding or through phosphorylation (P). TFs bind DNA and cause up- or down-regulation of gene expression. CAMTA, CaM-binding transcription activator; CBP60, CaM-binding protein; TGA, basic leucine zipper (bZIP) transcription factor; MYB, plant homologue of animal myeloblastosis TFs; WRKY, TF with conserved domain containing these amino acids. (Bickerton and Pittman 2012).
It has so far been difficult to prize apart the specific mechanisms which allow plants to respond to the host of concurrently occurring and often conflicting environmental stimuli. However, one of the best studied and understood examples of an external stimulus-induced Ca$^{2+}$ signalling response is that of guard cells stimulated with various abiotic components including the ‘stress’ hormone abscisic acic (ABA), CO$_2$, cold or H$_2$O$_2$ (Kim et al. 2010). For example, it is well established that stomatal closure induced by ABA is transduced by oscillations in cytosolic Ca$^{2+}$ (McAinsh et al. 1992). Furthermore, the magnitude and pattern of the cytosolic Ca$^{2+}$ oscillation, which can be manipulated by external application of Ca$^{2+}$, determines the degree of stomatal closure (McAinsh et al. 1995). Many of the molecular components that regulate the Ca$^{2+}$-dependent mechanisms of stomatal function are understood (Kim et al. 2010; Laanemets et al. 2013). For example, investigation of stomatal closure in the Arabidopsis det3 mutant guard cells revealed interesting insights into the role of ion transporters. Guard cells treated with Ca$^{2+}$, ABA, cold and H$_2$O$_2$, were shown to exhibit differential Ca$^{2+}$ signals in response to each stress, and each led to increases in stomatal closure (Allen et al. 2000). However, in det3 mutants defective in the vacuolar H$^+$-ATPase (V-ATPase), Ca$^{2+}$ oscillations in response to Ca$^{2+}$ and H$_2$O$_2$ were abolished, and stomatal closure was inhibited, while ABA and cold were still able to elicit Ca$^{2+}$ oscillations in det3, which led to stomatal closure akin to the wild type. The pH gradient generated by H$^+$ pumps such as the V-ATPase is required to energise H$^+$-coupled Ca$^{2+}$ exchangers (Barkla et al. 2008). The V-ATPase deficient det3 has therefore been proposed to be defective in vacuolar Ca$^{2+}$ sequestration, through inhibited CAX activity. The defective signalling of det3 mutants in response to Ca$^{2+}$ and H$_2$O$_2$, but not ABA and cold, highlights the complexity in plant Ca$^{2+}$ signal generating mechanisms, and the importance of multiple Ca$^{2+}$ transporters.
Figure 1.2: Ca\textsuperscript{2+} transporters in a typical plant cell

Ca\textsuperscript{2+-}permeable influx channels (orange circles), Ca\textsuperscript{2+-}ATPases (yellow hexagons), Ca\textsuperscript{2+}/H\textsuperscript{+} exchangers (CAX) (red pentagon) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (NCL) (green square) at the plasma membrane and internal membranes perform cellular Ca\textsuperscript{2+} homeostasis including maintaining resting cytosolic Ca\textsuperscript{2+} concentration, shape Ca\textsuperscript{2+} signals and refill Ca\textsuperscript{2+} stores. Black arrows indicate the direction of Ca\textsuperscript{2+} transport. ACA, autoinhibited Ca\textsuperscript{2+-}ATPase; CNGC, cyclic nucleotide gated channel; DACC, depolarisation activated Ca\textsuperscript{2+} channel; ECA, ER-type Ca\textsuperscript{2+-}ATPase; GLR, glutamate receptor; HACC, hyperpolarisation activated Ca\textsuperscript{2+} channel; IP\textsubscript{3}R-like, inositol-1,4,5-trisphosphate receptor-like channel; MCC, mechanosensitive Ca\textsuperscript{2+} channel; NAADPR, nicotinic acid adenine dinucleotide phosphate receptor channel; RRC, ROS-responsive channel; RyR-like, ryanodine receptor-like; TPC1, two-pore channel 1; VVCC, vacuolar voltage gated Ca\textsuperscript{2+} channel.
**Transporters in plant Ca\(^{2+}\) signalling:**

The requirement of plant cells to maintain \([\text{Ca}^{2+}]_{\text{cyt}}\) at a low resting concentration necessitates a plethora of transporters that allow movement of Ca\(^{2+}\) into and out of the cytosol (McAinsh and Pittman 2009). Ca\(^{2+}\)-permeable channels allow rapid influx of Ca\(^{2+}\) into the cytosol, while Ca\(^{2+}\) pumps and proton-coupled Ca\(^{2+}\) exchangers actively remove Ca\(^{2+}\). The presence of these Ca\(^{2+}\) transporters at both the plasma membrane and organellar membranes allows Ca\(^{2+}\) influx from intracellular stores, as well as from external sources (Figure 1.2).

**Ca\(^{2+}\) influx into the cytosol:**

A variety of Ca\(^{2+}\) permeable channels have been identified by electrophysiology assays and include hyperpolarisation activated channels (HACC), depolarisation activated channels (DACC), and voltage independent channels (VICC) (McAinsh and Pittman 2009). Several proteins have been also been identified with homology to known animal Ca\(^{2+}\) channels, including cyclic nucleotide gated channels (CNGC) and glutamate receptor-like channels (GLR) which are present at both the plasma membrane and that of organelles (McAinsh and Pittman 2009). Additionally, mechanosensitive channels (MCC), annexins, and reactive oxygen species (ROS) receptors (RRC), have more recently been suggested to play a role in Ca\(^{2+}\) signalling in plants (Swarbreck et al. 2013). Other candidates for cytosolic Ca\(^{2+}\) influx reside at organellar membranes, including the vacuole, such as the slow activating vacuolar channel (SV), and ligand gated channels such as inositol-1,4,5-trisphosphate receptors (IP\(_3\)R) and ryanodine receptors (R\(_y\)R), which as yet have not been characterised at the molecular level, nor have homology to similar receptors in animals, but nonetheless appear to be activated by similar ligands (Peiter 2011). Many of the aforementioned Ca\(^{2+}\)-permeable channels have been implicated in a variety of Ca\(^{2+}\) mediated stress-response pathways, some of which will be briefly summarised here.
**Ca^{2+}-permeable channels in Ca^{2+} signalling:**

Of all the Ca^{2+} influx channels to be characterised at the molecular level, CNGCs, which are encoded by 20 genes in *Arabidopsis* (Mäser et al. 2001), have the best documented role in Ca^{2+} mediated responses. CNGCs mediate Ca^{2+} influx into the cytosol following activation by cyclic nucleotides, including cAMP and cGMP, and are CaM regulated (Ali et al. 2006). CNGC10 of *Arabidopsis* has been shown to transport Ca^{2+}, as well as Mg^{2+} (Guo et al. 2010). Using antisense knockdown, this protein has also been shown to affect flowering time, leaf area and thickness, palisade length, gravitropism and starch accumulation (Borsics et al. 2007).

CNGC2 of *Arabidopsis* has been particularly interesting to study in terms of Ca^{2+} signalling. *Arabidopsis cngc2* mutants were shown to be hypersensitive to external Ca^{2+} but did not show any additional Ca^{2+} accumulation in roots, suggesting CNGC2 may be involved in signalling external Ca^{2+} concentration (Chan et al. 2003). Further analysis of *cngc2* mutants has revealed an important role of CNGCs in mediating plant responses to pathogen infection (Ma and Berkowitz 2011). Plants share a common feature in innate immunity to pathogens, involving localised cell death known as the hypersensitive response (HR). Induction of HR involves production of ROS following pathogen recognition, which leads to Ca^{2+} influx into the cytosol. The interaction of ROS with nitric oxide (NO) can also induce expression of defence-related genes, which also leads to HR. Ma et al. 2009 have presented a pathway in which pathogen reception leads to the generation of cAMP, which activates CNGC2 at the plasma membrane and thus Ca^{2+} influx into the cytosol, leading to production of ROS, NO, and HR (Figure 1.3). Evidence for this pathway has emerged from studies of *CNGC2* deficient *defence no death 1 (dnd1)* *Arabidopsis* mutants, which display constitutive systemic resistance (Clough et al. 2000). *CNGC2* deficient mutants also exhibit reduced NO production, while addition of an NO donor was able to restore HR (Ali et al. 2007). ROS generation in response to pathogen infection has also been shown to be almost absent in *dnd1* mutants, and impaired in
Arabidopsis treated with adenylyl cyclase inhibitors, which prevented the production of cyclic nucleotides (Ma et al. 2009). The same experiment showed a marked reduction in cAMP-mediated \([Ca^{2+}]_{cyt}\) in *dnd1* mutants, an effect which was mirrored in wild type *Arabidopsis* treated with an adenylyl cyclase inhibitor. CNGC2 therefore appears to play a significant role in the signal transduction of pathogen infection in *Arabidopsis*.

In addition to CNGCs, there exist 20 plant GLRs (Lacombe et al. 2001), which show high homology to animal ionotropic glutamate receptors (Lam et al. 1998), and display varied roles in plants. Addition of glutamate to *Arabidopsis* has been shown to increase *GLR3.4* expression 3- to 5-fold, and 5mM glutamate could induce a transient increase in \([Ca^{2+}]_{cyt}\) (Meyerhoff et al. 2005). *GLR3.4* expression was also induced 3- to 6-fold in response to touch or cold, respectively. Similarly, *GLR3.3* has also been shown to be activated by glutamate, as well as a host of other amino acids, which led to an increase in \([Ca^{2+}]_{cyt}\), an increase which was significantly reduced in a *glr3.3* knockout (Qi et al. 2006). Expression of GFP-tagged *AtGLR3.4* in human embryonic kidney (HEK) cells showed GLR3.4 localisation at the plasma membrane (Vincill et al. 2012), while in *Arabidopsis* and tobacco cells GLR3.4 has been shown to localise to both the plasma membrane and the chloroplast (Teardo et al. 2011). GLR3.4 has more recently been shown to interact with GLR3.2 in the correct initiation of lateral root development in *Arabidopsis*. GLR3.2 and GLR3.4 were shown to localise mainly to the sieve plates of phloem, and mutations in either protein, or both proteins concurrently, presented the same increased production and differential placement of lateral root primordia compared to wild type plants (Vincill et al. 2013).
Figure 1.3: A representative pathogen-mediated Ca\textsuperscript{2+} signalling pathway

Binding of an elicitor (AtPep) to a receptor (AtPepR1) activates adenylyl cyclase (AC) and/or guanylyl cyclase (GC), which generate cAMP or cGMP, respectively. Increase in cAMP and cGMP concentration activates cyclic nucleotide gated channels (CNGC), which causes influx of Ca\textsuperscript{2+} into the cytosol. Ca\textsuperscript{2+} binding to calmodulin (CaM) and calcium-dependent protein kinases (CDPK) indirectly activates the hypersensitive response (HR), defence-related gene expression and increased salicylic acid (SA) production. CaM activates NO synthase, producing NO, which acts in conjunction with \textsubscript{H}_2\textsubscript{O}_2 generated from CDPK-activated NADPH oxidase in order to stimulate HR and gene expression. CaM binding to transcription factors (TF) (e.g. CAMTA, WRKY and CBP60) activates gene expression, while CDPK may phosphorylate TFs which can then bind to DNA (dashed arrows). Red line depicts inhibition of CNGC activity by Ca\textsuperscript{2+}/CaM.

(Bickerton and Pittman 2012)
Importance of efflux proteins in shaping Ca\(^{2+}\) signals:

Considering the nature of the shape of Ca\(^{2+}\) signals, the Ca\(^{2+}\) efflux proteins are of particular interest, in both a capacity to mediate cytosolic Ca\(^{2+}\) signals, and in maintenance of Ca\(^{2+}\) stores, both intracellular and apoplastic. The two most studied families of Ca\(^{2+}\) efflux proteins are Ca\(^{2+}\)-ATPases and CAXs (McAinsh and Pittman 2009, Pittman et al. 2011, Spalding and Harper 2011). Ca\(^{2+}\)-ATPases generally show high affinity, low capacity transport of Ca\(^{2+}\) out of the cytosol, while CAXs sequester Ca\(^{2+}\) away from the cytosol with low affinity but high capacity transport. It has been proposed that the high affinity of Ca\(^{2+}\)-ATPases for Ca\(^{2+}\) may render them more important in maintaining Ca\(^{2+}\) homeostasis, while the high capacity nature of CAX perhaps suggests an important role in mediating Ca\(^{2+}\) signals during transient large increases and oscillations (Hirschi 2001). Recent evidence from *Arabidopsis* also suggests the presence of a novel type of Na\(^+\)/Ca\(^{2+}\) exchanger at plant plasma membranes, which may play a role in mediating plant abiotic stress responses.

**Ca\(^{2+}\)-ATPases:**

Plants contain two types of Ca\(^{2+}\)-ATPase which group together based on homology with animal Ca\(^{2+}\) pumps. In *Arabidopsis*, there are 4 endoplasmic reticulum-type Ca\(^{2+}\)-ATPases (ECA) which are analogous to animal sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA), while the 10 autoinhibited Ca\(^{2+}\)-ATPases (ACA) in *Arabidopsis* are analogous to animal plasma membrane Ca\(^{2+}\)-ATPases (PMCA). Ca\(^{2+}\)-ATPases have well-documented roles in maintaining ion homeostasis and have also been implicated in a variety of abiotic stress responses. For example, *Arabidopsis* ACA2 has been shown to rescue salt-hypersensitive Ca\(^{2+}\) pump-deficient yeast, which was attributed to the restoration of cytosolic Ca\(^{2+}\) oscillations in ACA2-transformed cells, leading to increased salt tolerance (Anil et al. 2008). Expression studies in rice also point towards a role for both ECAs and ACAs in mediating salt stress, as expression of *OsECA1* and *OsACA5* increases in salt-treated plants (Singh et al. 2014). Expression of *OsACA4* was also
observed to be 2 to 3-fold higher in a salt tolerant variety of rice, compared to that of a salt-sensitive variety (Yamada et al. 2014). Other abiotic stimuli in which Ca\(^{2+}\)-ATPases have been implicated include plant responses to touch (Klüsener et al. 1995), cold (Puhakainen et al. 1999) and heat (Liu et al. 2008). Additionally, there exists strong evidence of a role for Ca\(^{2+}\)-ATPases in mediating plant responses to biotic stimuli, namely in regulating programmed cell death (PCD) in response to pathogen infection. For example, double knockout of *Arabidopsis* ACA4 and ACA11 led to a salicylic acid (SA)-dependent increase of PCD in transformed cells, which could be rescued by co-transformation with ACA11 (Boursiac et al. 2010). Furthermore, RNAi silencing of ECA NbCA1 in tobacco led to an increased incidence of pathogen-mediated PCD in knockdowns (Zhu et al. 2010). Elevated [Ca\(^{2+}\)]\(_{cyt}\) spikes greater than wild type were observed in mutants in response to the fungal elicitor cryptogein, which suggests a role for ECAs in Ca\(^{2+}\) mediated signalling of PCD. The fact that ACAs can be activated by calmodulin (CaM), a Ca\(^{2+}\) binding protein involved in Ca\(^{2+}\) signal transduction, further points toward a role for Ca\(^{2+}\)-ATPases in mediating plant stress responses (Hwang et al. 1997).

**Ca\(^{2+}\)/H\(^{+}\) exchangers (CAX):**

CAXs are a ubiquitous type of ion transporter, found throughout prokaryotes and eukaryotes, and related to other ion-coupled cation exchangers, including the Na\(^{+}/\text{Ca}\(^{2+}\) exchangers (NCX), which are all members of the CaCA transporter superfamily (Emery et al. 2012). CAXs are widely present throughout plants, protists, bacteria, cyanobacteria, fungi, algae and some animals, although exceptions include mammals and insects (Manohar et al. 2011). Phylogenetic analysis reveals three types of CAX; type I, which includes plant, fungal, bacterial and protist CAX, type II found in fungi and some animals, and bacterial type III CAX (Shigaki et al. 2006, Emery et al. 2012). Apart from in the yeast *Saccharomyces cerevisiae*, to date CAXs have been mostly studied from higher plants, and mostly in *Arabidopsis* and rice. Plant CAXs were first characterised using yeast when
Arabidopsis CAX1 and CAX2 were shown to rescue the Ca hypersensitivity phenotype of yeast mutants deficient in vacuolar Ca\(^{2+}\) sequestration (Hirschi et al. 1996). Similar suppression of yeast Ca sensitivity was shown with CAXs from other plants like mung bean and rice (Ueoka-Nakanishi et al. 2000, Kamiya et al. 2005). CAX1 was later shown only to rescue the Ca hypersensitivity phenotype upon truncation at the N-terminus, which revealed an N-terminal autoinhibitory domain that appears to be common to many plant and non-plant CAXs (Pittman and Hirschi 2001, Pittman et al. 2002b, Pittman et al. 2009, Guttery et al. 2013). Subsequent genetic analysis of CAXs has confirmed their role as vacuolar Ca\(^{2+}/H^+\) exchangers in planta. Arabidopsis cax1 mutants showed a 40% reduction in capacity of Ca\(^{2+}/H^+\) transport at vacuolar membrane-enriched vesicles, while Ca\(^{2+}\)-ATPase activity increased compared to wild type (Cheng et al. 2003). It appears that there is a certain degree of CAX redundancy, as cax1 mutants showed increased transcript abundance of CAX3 and CAX4 (Cheng et al. 2003, Conn et al. 2011). Similarly, in other CAX mutants CAX gene induction has been observed (Connorton et al. 2012), highlighting the importance of a functional Ca\(^{2+}/H^+\) exchanger in a plant cell, which is further demonstrated by the severe phenotype of mutant plants lacking multiple CAX isoforms (Cheng et al. 2005).

In addition to functioning as Ca\(^{2+}/H^+\) exchangers, it is clear that many plant CAX proteins can transport a range of cations including Ca\(^{2+}\), Cd\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\), as demonstrated through yeast expression (Shigaki et al. 2003) and from plant analysis (Koren'kov et al. 2007; Connorton et al. 2012), and can thus provide tolerance to metal stress through direct cation sequestration (Hirschi et al. 2000, Wu et al. 2011). Hence CAX can also be defined as a cation/H\(^+\) exchanger. It has also been shown that when CAX1 and CAX3 are co-expressed they can confer salt tolerance in yeast, possibly due to Na\(^+\)/H\(^+\) exchange activity (Zhao et al. 2009b). In contrast, some of the altered cation stress phenotypes observed in CAX knockout mutants might be due to indirect Ca\(^{2+}\) homeostasis effects. For example, increased Mg tolerance in cax1 and cax1/cax2
mutants does not appear to be due to direct Mg\textsuperscript{2+} transport but due to alterations in cellular Ca\textsuperscript{2+} (Connorton et al. 2012). In *Arabidopsis* there are 6 CAX genes, which cluster into two phylogenetic groups; type IA (CAX1, CAX3 and CAX4) and type IB (CAX2, CAX5 and CAX6). CAX1 and CAX3 appear to be important for Ca\textsuperscript{2+} homeostasis (Cheng et al. 2003; Conn et al. 2011), while CAX2 and CAX5 have a preference towards Mn\textsuperscript{2+} transport (Shigaki et al. 2003, Pittman et al. 2004, Edmond et al. 2009). However, there is not a clear-cut distinction between the groups based on substrate. CAX2 and CAX5 can also transport Ca\textsuperscript{2+} (Edmond et al. 2009) while CAX4 is transcriptionally induced by various metals (Cheng et al. 2002) and cax4 knockdown mutants show sensitivity to metal stress (Mei et al. 2009). There may also be other physiological functions of CAX transporters. CAX1 and CAX3 also play an important role in gas exchange and plant productivity, as well as for regulating apoplastic Ca concentration (Conn et al. 2011). In addition, these transporters have been implicated in the signalling of root-shoot phosphate transport in response to phosphate starvation (Liu et al. 2011), as well as regulation of apoplastic pH (Cho et al. 2012), which provides an interesting insight into the role for CAX in whole plant signalling. The diverse roles for CAX in the sequestration and homeostasis of Ca\textsuperscript{2+}, as well as providing tolerance to transition metal and salt stress, suggests CAXs are of great interest when elucidating plant responses to abiotic stress.

**Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (NCX):**

Until recently it was thought that only Ca\textsuperscript{2+}-ATPases and H\textsuperscript{+}-coupled Ca\textsuperscript{2+} exchangers were responsible for Ca\textsuperscript{2+} extrusion from the cytosol in plant cells, but that Na\textsuperscript{+}-coupled Ca\textsuperscript{2+} exchangers, which are prevalent in animal cells and encoded by NCX genes (Emery et al. 2012) were absent in higher plants. However, recent evidence suggests a role for Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (NCX) at the plasma membrane of *Arabidopsis* cells (Wang et al. 2012). The *Arabidopsis* NCX-like protein (AtNCL) shows structural and sequence similarity with CAX proteins, with regions of homology at the C-terminus and, like CAX,
10-11 transmembrane domains (Shigaki et al. 2006). AtNCL has two Ca\(^{2+}\)-binding EF-hands suggesting that the protein may be regulated by Ca\(^{2+}\) (Emery et al. 2012). Recent phylogenetic analysis demonstrated that *Arabidopsis* has at least two NCL genes (also named “EF-CAX,”) and showed that related genes are prevalent in the genomes of high plants, moss and algae, and these genes are mostly related to higher plant CAX genes rather than any other exchanger families (Emery et al. 2012). When expressed in yeast, *AtNCL* was unable to suppress the yeast mutant Ca hypersensitive phenotype and did not appear to possess H\(^{+}\)/Ca\(^{2+}\) exchange activity but did show Na\(^{+}\)/Ca\(^{2+}\) exchange activity when expressed in mammalian cells (Wang et al. 2012). AtNCL-YFP fusion proteins localised to the *Arabidopsis* plasma membrane rather than the vacuole and *AtNCL* expression was induced in response to a number of abiotic stimuli including salt, ABA, cold and heat. Furthermore, *atncl* knockout lines were less sensitive to salt stress compared to wild type, and had reduced Na and increased Ca content compared to wild type under both normal conditions and salt stress, indicating that AtNCL functions in Ca\(^{2+}\) efflux at the plasma membrane (Wang et al. 2012). It appears that AtNCL represents a novel type of plant Ca\(^{2+}\) transporter, which may provide an interesting area for study particularly in terms of Ca\(^{2+}\) efflux in response to salt stress.

**The physiological functions of plant CAXs:**

Plant CAXs have been demonstrated to play a role in a variety of physiological functions (Figure 1.4). Genetic studies have highlighted the importance of CAX in maintaining Ca\(^{2+}\) homeostasis in plants. In tobacco plants over-expressing *Arabidopsis CAX1*, Ca accumulation was 30% higher in leaves compared with a vector control, and was double in roots (Hirschi 1999). The transgenic plants showed a number of symptoms reminiscent of Ca\(^{2+}\) deficiency, such as sensitivity to K and Mg, and cold shock, while these were reversed upon application of Ca (Hirschi 1999). To complement this phenotype, it was more recently shown that *cax1* mutants could tolerate growth in serpentine solutions with a low Ca/Mg ratio, while mutants grew poorly in higher concentrations of Mg (Bradshaw
A similar phenotype was observed when CAX1 was over-expressed in tomato in order to increase total fruit Ca levels by vacuolar sequestration, however, the CAX1 over-expressing tomatoes showed an increased incidence of blossom-end rot which is associated with localised cellular Ca$^{2+}$ deficiency (Park et al. 2005). Due to the high level of Ca$^{2+}$ sequestration performed by CAX1, attempts have been made to biofortify other crops, including carrots (Morris et al. 2008), as Ca deficiency remains one of the leading causes of malnutrition worldwide.

CAX1 is highly expressed in Arabidopsis leaves, while CAX3 shows highest expression in roots, but analysis of cax1cax3 mutants indicates functional interaction between the two proteins in mediating ion homeostasis in planta (Cheng et al. 2005), which is explained both by genetic redundancy (Conn et al. 2011) and direct protein-protein interaction between both CAXs (Zhao et al. 2009a, Zhao et al. 2009b). As might also be expected, total Ca content is reduced slightly in cax1 plants and substantially in cax1cax3 plants (Catalá et al. 2003, Cheng et al. 2005, Conn et al. 2011). Furthermore, the Mg content of cax1cax3 shoots was also lower than control, and interestingly P, Mg and Zn levels were higher. It is therefore unclear whether the severe growth defects such as leaf-tip necrosis, stunted growth, and small siliques exhibited by the cax1cax3 plants are partly due to impaired Ca$^{2+}$ homeostasis or alteration in concentration of other metals (Cheng et al. 2003). Further probing into the P accumulation phenotype revealed that cax1cax3 mutants not only accumulate more P but also show a greater shoot/root P ratio under P starvation (Liu et al. 2011). CAX1 expression levels were significantly reduced in shoots upon P starvation. Furthermore, the expression of phosphate starvation response genes was greatly altered in cax1cax3 mutants under high P, which included up-regulation of several phosphate signalling genes, therefore implicating CAX1 and CAX3 in root-shoot signalling of P starvation in Arabidopsis (Liu et al. 2011). Another interesting gene up-regulated in cax1cax3 mutants is a glucose-6-phosphate/phosphate translocator GPT2 (Liu et al. 2011), which is thought to have a role in dynamic acclimation of
photosynthesis in response to environmental perturbations (Athanasiou et al. 2010), highlighting the variety of processes that are potentially mediated by CAX.

While CAX1 and CAX3 show differential tissue-specific expression in mature Arabidopsis, both genes are expressed in Arabidopsis seeds (Zhao et al. 2009b, Punshon et al. 2012). However, CAX1 has highest expression in the seed coat, in the cotyledon and at the heart stage of development, as well as in all stages of chalazal endosperm development, while CAX3 showed mostly moderate expression, but was highly expressed in the linear cotyledon and mature green seeds. An analysis of the distribution of various elements in seeds found that cax1 mutants had higher Ca, Cu and Mn content, while CAX1 over-expressing seeds showed a reduction in these elements, and cax3 showed similar perturbances in Ca and Cu (Punshon et al. 2012). Using SXRF microtomography to further probe elemental distribution in seeds, it was found that Ca content was much higher in the seed coat of cax1, cax3 and cax1cax3 than wild type, while the opposite occurred in 35S::CAX1 seeds (Punshon et al. 2012). Furthermore, Zn, K and Ca were higher in cax1 and cax3 seeds, but lower in those of 35S::CAX1. In endodermal cells of cax1cax3 mutants, vacuolar storage of Ca, Cu, Fe, P, S and Zn was completely absent (Punshon et al. 2012). A significantly higher accumulation of Mn and Zn has also been measured in cax2 seeds, while cax2, cax3, cax1cax2 and cax2cax3 mutants all showed increased Fe, K and P (Connorton et al. 2012). The challenge is now to dissect the elemental changes that are due to direct ion transport by CAXs and those that are indirect effects. Perturbed seed metal homeostasis has been linked to significant reduction in germination in all mutants, although the strongest decrease was observed in cax3 seeds, which showed only 25% germination compared with over 80% in wild type plants (Connorton et al. 2012).

In mesophyll cells, CAX1 and CAX3 appear to be especially important in maintaining Ca$^{2+}$ homeostasis, as well as physiological processes such as stomatal conductance. Ca content in mesophyll cells is significantly reduced in cax1cax3 mutants,
while the Ca$^{2+}$-ATPase mutant *aca4aca11* shows no change in Ca content, which suggests that CAX is the main route of Ca$^{2+}$ sequestration into mesophyll vacuoles (Conn et al. 2011). Interestingly, *cax1cax3* mutants show a decrease in stomatal aperture closure compared to wild type plants (Conn et al. 2011), which was also observed to occur in *det3* mutants lacking V-ATPase activity (Allen et al. 2000). The control of CAX by V-ATPases has been well established (Krebs et al. 2010), therefore CAX1 and CAX3 may regulate stomatal aperture via guard cell Ca$^{2+}$ signalling. However, it has also been argued that a 4-fold increase in apoplastic Ca in the mesophyll of *cax1cax3* plants is the cause of stomatal aperture closure, leading to lower stomatal conductance, CO$_2$ limitation and thus reduced growth (Conn et al. 2011). It has also been suggested that a modulation of apoplastic pH in guard cells by CAX1 and CAX3, probably via plasma membrane H$^+$-ATPase activity, influences auxin transport activity and in turn regulates stomatal aperture closure (Cho et al. 2012).
Leaves & shoots:
- Mesophyll Ca$^{2+}$ sequestration
- Ion homeostasis
- Nutrient uptake, e.g. PO$_4^{3-}$
- Growth and development
- Maintenance of apoplastic Ca$^{2+}$ and pH
- Guard cell stomatal aperture

Seeds & embryos:
- Mineral accumulation
- Strong expression of CAX1 and CAX3 in developing embryos
- cax1/cax2, cax1/cax3 - germination

Phosphate signalling:
- cax1cax3 - up-regulation of P$_i$ signalling genes
- CAX1 and CAX3 - altered expression in shoots and roots upon P$_i$ starvation

Roots:
- cax1 - reduced root growth
- CAX2 - uptake of Mn$^{2+}$ and Cd$^{2+}$
- CAX3 - P$_i$ starvation
- CAX4 - metal tolerance

Figure 1.4: Physiological roles of Ca$^{2+}$/H$^+$ exchangers (CAX) in plants

Red and brown boxes indicate the highest expression of various *Arabidopsis* and *Oryza sativa* (rice) CAX genes, respectively. Yellow boxes indicate where *Arabidopsis* CAX has been implicated in plant development and stress response, which are described in bullet points on the right, adjacent to their position on the plant.
CAX in abiotic stress signalling:

A number of studies have implied a role for Arabidopsis CAX in mediating plant abiotic stress responses, particularly salt and cold stress. Expression of CAX3 and CAX4 were slightly up-regulated in wild type plants and markedly in cax1 mutants under salt stress (Cheng et al. 2003). CAX2 also showed slight induction in response to salt stress but with no difference in wild type and cax1 plants. Interestingly the vacuolar Ca\textsuperscript{2+}-ATPase ACA4 was down-regulated in response to high salt suggesting that CAXs are more important in vacuolar Ca\textsuperscript{2+} sequestration under salt stress conditions. CAX1 and CAX3 display functional interplay in a variety of processes, and their co-expression confers salt tolerance in yeast (Zhao et al. 2009b). However, some distinct roles have been observed for CAX3. In guard cells, for example, CAX3 expression increases between 2.5 and 4.9-fold in response to ABA treatment (Leonhardt et al. 2004), while cax3 plants show the most reduced growth on ABA (Connorton et al. 2012). An analysis of cax3 and cax1cax3 mutants showed that these plants have severely reduced root growth when treated with NaCl compared with both wild type and cax1 plants (Zhao et al. 2008). Furthermore, Ca\textsuperscript{2+}/H\textsuperscript{+} exchange activity was also decreased in cax3 but not cax1 mutants under salt stress. Therefore, the genetic evidence suggests that in Arabidopsis CAX activity is important in the salt stress response, with CAX3 appearing to be the most important isoform.

Slightly conflicting therefore is evidence from yeast expression studies implicating Arabidopsis CAX1 as a component in a key Ca\textsuperscript{2+}-regulated salt stress response pathway. The CIPK-type protein kinase SOS2 (also known as CIPK24) is a central component in the salt overly sensitive (SOS) cell signalling response pathway to excess salt. The Ca\textsuperscript{2+}-binding CBL-type sensor SOS3 (CBL4) activates SOS2, and together this SOS2/SOS3 CBL/CIPK complex activates SOS1, an Na\textsuperscript{+}/H\textsuperscript{+} exchanger at the plasma membrane (Ji et al. 2013). In a yeast assay, SOS2 was shown to activate CAX1 Ca\textsuperscript{2+}/H\textsuperscript{+} exchange through direct protein interaction (Cheng et al. 2004). It was not confirmed whether SOS2
activates CAX1 by phosphorylation, but when a candidate phosphorylated Ser residue (Ser-25) within the CAX1 N-terminus (Pittman et al. 2002a) is mutated (to S25A), SOS2 activation cannot occur. The regulation by SOS2 was also found to be independent of SOS3 (Cheng et al. 2004). An interaction between these two proteins highlights a potentially important role for CAX1 in the signalling of salt stress, mediated in part by the SOS pathway, but interaction in planta has not been demonstrated. Further evidence of a joint role for these proteins in mediating salt stress comes from knockdown and overexpression studies of SAD1, which modulates the splicing of stress specific proteins (Cui et al. 2014). In sad1 mutants, aberrant splicing was observed in CAX1 and SOS2, as well as salt and cold stress related transcription factors, while SAD1 overexpressing Arabidopsis was found to be more tolerant to salt, due to an increase in splicing efficiency and inhibition of alternative splicing (Cui et al. 2014).

Studies in other plant species such as rice, tobacco, soybean, and the halophyte Suaeda salsa have further supported the role of CAX in salt stress. Expression of SsCAX1 from S. salsa increases sensitivity to salt in transgenic Arabidopsis (Han et al. 2012). Likewise, expression of deregulated Arabidopsis CAX1 in tobacco increases salt sensitivity (Mei et al. 2007). This may indicate that alteration in net CAX activity causes a disruption in the plant’s ability to mediate a normal salt stress response, possibly through disruption in modulation of the salt-induced Ca\(^{2+}\) signature. In contrast, high expression of a CAX gene was observed in a salt-tolerant rice variety under salt stress conditions, while rice CAX expression was reduced in salt-sensitive varieties (Senadheera et al. 2009). Moreover, in transgenic rice, over-expression of a V-ATPase subunit from Spartina alterniflora, a halophyte grass, increased salt tolerance (Baisakh et al. 2012). This coincided with an increase in Ca\(^{2+}\) sequestration and a substantial up-regulation of several genes involved in the salt stress response, pointing toward a role for CAX in mediating salt tolerance in rice. Similarly, expression of a soybean CAX (GmCAX1) was induced by various treatments including salt, and also ABA, PEG, Ca and Li, and when GmCAX1 was
over-expressed in *Arabidopsis*, greater tolerance to both Li and Na was conferred, while the plant accumulated less Na, K and Li (Luo et al. 2005). Interestingly, GmCAX1 was suggested to be localised to the plasma membrane rather than the tonoplast, indicating that GmCAX1 caused decreased salt stress sensitivity by cellular Na$^+$ efflux, potentially by this exchanger. The first clear-cut evidence of a CAX from a photosynthetic organism being able to directly transport Na$^+$ has come from the green alga *Chlamydomonas reinhardtii*. When heterologously expressed in yeast, CrCAX1 can transport Na$^+$ via H$^+$ exchange into yeast vacuoles (Pittman et al. 2009).

CAX1 has been implicated in controlling the expression of *CBF/DREB1* genes, which are induced under cold stress in *cax1* mutants (Catalá et al. 2003). CAX1 is transiently inducible under cold stress in *Arabidopsis* leaves. However, expression of CAX1 in response to cold was shown to be independent of ABA, as expression was equivalent in ABA-deficient and ABA-insensitive mutants, and in wild type plants (Catalá et al. 2003). More interestingly, *cax1* mutants showed increased tolerance to cold-acclimated freezing stress, coincident with induction of known cold responsive genes by low temperature. CBF/DREB1 is a class of transcription factor that binds to specific cis-acting genetic elements (DRE; drought responsive cis-element) in abiotic stress response genes (Katiyar-Agarwal et al. 2006). *CBF/DREB1* transcription factors are up-regulated in response to a variety of abiotic stimuli, including dehydration, cold, and salt (Narusaka et al. 2003). In *cax1* plants, under cold stress, the transcription factors *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A* were more highly expressed than in wild type plants (Catalá et al. 2003). CAX1 therefore appears to be a negative regulator of the cold acclimation response, with the possible conclusion that Ca$^{2+}$ signals modulated by CAX1 are required for proper generation of the cold response via transcription factor induction. In contrast, a CAX from cotton (GhCAX3), which can be up-regulated by cold stress (as well as salt stress and ABA) positively confers a degree of cold tolerance when over-expressed in yeast and mature plants, although the *GhCAX3* over-expression lines confer
cold sensitivity on seed germination (Xu et al. 2013). Ca\(^{2+}/H^+\) exchangers therefore appear to play an important role in the signal transduction of cold stress, but there are differences between species, potentially due to the difference in expression pattern and species-specific signal transduction pathways.

Another potential signalling role for CAX is indicated by the tight relationship between CAX and pH, as pH modulation, like Ca\(^{2+}\) modulation, may mediate intracellular signalling processes (Felle 2001). There is increasing evidence that ion transporters like CAX that mediate H\(^+\) exchange play a role in pH homeostasis within cells (Pittman 2012). In addition, CAX1 and CAX2 activity can in turn be regulated by cytosolic pH (Pittman et al. 2005). Furthermore, there exist various examples of deregulation of V-ATPase activity in CAX mutants, while reduced activity of the plasma membrane H\(^+\)-ATPase has also been observed in cax3 mutants (Barkla et al. 2008, Zhao et al. 2008). Recently there has been evidence of a link between CAX1/CAX3 activity, apoplastic pH in guard cells and IAA-mediated stomatal closure. Arabidopsis cax1cax3 mutants failed to maintain apoplastic pH compared to wild type, which was observed alongside reduced inhibition of ABA-mediated signalling of stomatal closure by IAA (Cho et al. 2012). An analysis of gene expression in Arabidopsis roots showed up-regulation of a number of genes involved in Ca\(^{2+}\) signalling in response to a lowering of pH, corresponding with over-expression of CaM-motifs in the promoters of pH responsive genes (Lager et al. 2010). Additionally, evidence suggests that pH and Ca\(^{2+}\) are modulated both in the cytosol and the apoplast in response to abiotic stresses, including salt and drought (Gao et al. 2004). Thus, the close relationship observed between CAX and pH indicates another link and potential mechanism in mediating plant abiotic stress responses.

**Role of CAX in mediating plant responses to toxic metal stress:**

Although CAX2 has been shown to transport Ca\(^{2+}\) into yeast vacuoles (Hirschi et al. 1996), cax2 mutants of Arabidopsis do not show any alteration in vacuolar Ca\(^{2+}/H^+\) activity or any Ca sensitivity defects (Pittman et al. 2004). Vacuolar Mn\(^{2+}/H^+\) exchange is,
however, reduced in cax2 plants. Arabidopsis cax2 and cax2cax3 mutants were more recently shown to exhibit vastly reduced growth on high Mn (Connorton et al. 2012). CAX2 contains a 3 amino acid residue which confers Mn$^{2+}$ specificity, removal of which inhibits Mn$^{2+}$ transport by CAX2 (Shigaki et al. 2003). Over-expression of CAX2 in tobacco gives increased accumulation of Ca, Cd and Mn, as well as tolerance to growth on high Mn, which points toward a role for CAX2 in sequestering heavy metals and ameliorating heavy metal stress (Hirschi et al. 2000). Expression of CAX5, a close homologue of CAX2, has been shown to increase under high Cd and Mn stress (Edmond et al. 2009). In yeast, CAX5 functions similarly to CAX2, whereby N-terminal truncation led to transport of Ca$^{2+}$ and Mn$^{2+}$ into yeast vacuoles. Additionally, the coincident expression of CAX2 and CAX5 in all plant tissues, including highest expression in the roots, suggests that these proteins play similar and potentially redundant roles in transporting transition metals such as Mn$^{2+}$ and Cd$^{2+}$ (Edmond et al. 2009). As yet the phenotype of cax5 mutants has not been examined and it is unknown whether Mn$^{2+}$/H$^{+}$ exchange would be reduced in these plants. Furthermore, cax2cax5 double mutants may indicate whether there is any functional interplay and/or redundancy between CAX2 and CAX5, as there is between CAX1 and CAX3.

Arabidopsis CAX4 has high sequence identity with CAX1, yet CAX4 was unable to suppress the Ca$^{2+}$ hypersensitivity of the yeast mutant and so direct evidence of CAX4 Ca$^{2+}$ transport has been lacking (Cheng et al. 2002). Although in planta analysis indicates that CAX4 can mediate vacuolar Ca$^{2+}$/H$^{+}$ exchange (Mei et al. 2009), this CAX may be more important for transport of other metals. CAX4 is expressed at a low levels throughout Arabidopsis plants, although expression increases in response to Mn, Ni and Na conditions (Cheng et al. 2002). In cax4 mutants, root length and lateral root number is reduced in response to high levels of Cd and Mn, and this phenotype is reversed upon complementation with wild type CAX4 (Mei et al. 2009). Furthermore, tobacco plants over-expressing CAX2 and CAX4 show increased transport of Zn$^{2+}$ and Cd$^{2+}$ at the tonoplast,
as well as increased root Cd content (Koren'kov et al. 2007). Furthermore, these plants show higher tolerance to growth on excess Zn, Mn and Cd.

Due to the observed effects of selected CAX proteins for providing tolerance to metal stress in plants through the vacuolar sequestration of toxic ions such as Mn$^{2+}$, Cd$^{2+}$ and Zn$^{2+}$, there has been some interest in modifying plants in order to bioremediate toxic soils. To this end, a mutant of CAX1 displaying high Cd$^{2+}$ transport was over-expressed in petunia plants and conferred greater tolerance to growth on high levels of Cd (Wu et al. 2011). Transgenic plants were also shown to accumulate significantly greater levels of Cd, while plant development was unaffected until the flowering stage, which suggests that the over-expression of the CAX mutant may provide a useful route into remediating Cd-enriched soils.

**The use of *C. reinhardtii* as a model to further elucidate the role of CAX proteins:**

The precise role of CAX in modulating plant Ca$^{2+}$ signals has been hard to discern, potentially due to CAX gene redundancy in higher plants and the presence of other important Ca$^{2+}$ efflux proteins, including Ca$^{2+}$ pumps and NCX. This is in contrast to model organisms, such as *S. cerevisiae* or *Plasmodium*, which possess just one or two CAX genes and in which genetic analysis of CAXs have been very fruitful (Denis and Cyert 2002, Guttery et al. 2013). A photosynthetic model organism with great potential for further elucidating the role of CAX in cell signalling is *C. reinhardtii*. Compared to higher plants there is a lesser degree of gene redundancy in *Chlamydomonas*, which contains only two expressed CAX open reading frames compared with six in *Arabidopsis* and rice (Emery et al. 2012).

Phylogenetic analysis of CAX proteins in *Chlamydomonas* shows that CrCAX1 and CrCAX2 cluster with type IC CAXs, which includes the protozoan *T. falciparum* and the red alga *T. pseudonana* (Pittman et al. 2009). Although distinct from the phylogenetic sub-groups containing higher plant CAXs, these proteins share many features of the
higher plant CAXs, including a similar post-translational regulatory mechanism, tonoplast localisation, and Ca\(^{2+}/H^+\) exchange activity (Pittman et al. 2009). CrCAX1 is also interesting as it can transport a diverse range of cations including Na\(^+\). The ability of *Chlamydomonas* CAX1 to transport Na\(^+\) provides an interesting target to further study the role of CAX in mediating salt stress, both in a direct Na\(^+\) homeostasis and Ca\(^{2+}\) signalling capacity, especially considering that when over-expressed in *Arabidopsis*, CrCAX1 has been shown to increase plant salt tolerance (Pittman et al. 2009). As *Chlamydomonas* CAX1 can transport Cd\(^{2+}\), as well as Ca\(^{2+}\) and Na\(^+\) (Pittman et al. 2009), further research into over-expression of CrCAX1 in algae may lead to developments in the bioremediation of polluted ground waters, ponds and lakes.

**Ca\(^{2+}\) signalling in algae:**

The best-characterised role for Ca\(^{2+}\) in signalling cellular responses in algae has been gleaned from the seaweed *Fucus serratus*, a marine brown algae, eggs of which were shown to exhibit a transient increase in cytosolic Ca\(^{2+}\) concentration as a result of fertilisation initiation (Roberts et al. 1994). Although a whole-cell Ca\(^{2+}\) increase was not necessary for *Fucus* egg activation, external Ca\(^{2+}\) was required in order to initiate early activation events, including cell wall exocytosis. Further probing of this phenotype revealed that Ca\(^{2+}\) influx was the main driver of such early-activation associated events, as IP\(_3\)-stimulated cytosolic Ca\(^{2+}\) influx could only cause cell wall exocytosis at the site of microinjection (Roberts and Brownlee 1995).

Analysis of *Fucus* rhizoids has also highlighted the role for Ca\(^{2+}\) transients in signalling external stresses, as osmotic stresses of increasing magnitude have been shown to elicit Ca\(^{2+}\) transients which increase with the strength of the applied stimulus (Goddard et al. 2000). Localised release of caged IP\(_3\) in *Fucus* rhizoids was shown to increase cytosolic Ca\(^{2+}\) concentration both at the apex and nuclear region and caused a wave of cytosolic Ca\(^{2+}\) influx in either case (Goddard et al. 2000), although this was not
directly linked to application of osmotic stress. Hyperosmotic stress-induced Ca$^{2+}$ signalling in *Fucus* rhizoid cells was later shown to form as a wave, generated at the cell apex then spreading throughout the cell, which relies on ROS production in order to generate the initial, apical Ca$^{2+}$ increase (Coelho et al. 2002). More recently, the involvement of Ca$^{2+}$ transients in the correct polarisation and progression of the cell cycle has also been demonstrated in *Fucus* (Bothwell et al. 2008, Coelho et al. 2008). Together, these results highlight the importance and necessity of Ca$^{2+}$ signalling in *Fucus serratus* in responding to external stresses, and in co-ordinating developmental processes, as well as the broad range of processes which are coordinated by Ca$^{2+}$ dependent mechanisms in a single organism.

**Ca$^{2+}$ signalling in *Chlamydomonas*:**

Calcium signals have been suggested to play a role in a variety of *Chlamydomonas* responses, including the much-studied mating reaction (Quarmby 1994). The local anaesthetic lidocaine (which blocks Na$^+$ channels), for example, was shown to inhibit cell fusion in mating *C. reinhardtii* cells, by blocking the uptake of external Ca$^{2+}$, suggesting that Ca$^{2+}$ might play a role in sex signalling (Snell et al. 1982). Fertilisation in *C. reinhardtii* was also associated with increased Ca$^{2+}$ efflux, which hinted at an increased accumulation of Ca$^{2+}$ in external media (Bloodgood and Levin 1983). To further support these observations, X-ray maps of bound Ca$^{2+}$ in *C. reinhardtii* gametes showed arrangement of Ca$^{2+}$ into distinct granules before mating, which then became diffuse throughout the cell during mating, suggesting a release of Ca$^{2+}$ from internal stores forms part of the sex signalling response in *Chlamydomonas* (Kaska et al. 1985). Analysis of mating structure formation in *C. eugametos*, a close relative of *C. reinhardtii*, provided further evidence for Ca$^{2+}$ signalling of the mating response, as mating structures were found to form in response to a number of compounds which are known to increase [Ca$^{2+}$]$_{cyt}$, including IP$_3$ (Schuring et al. 1990). However, Goodenough et al. (1993) found that IP$_3$ levels remain constant during the *C. reinhardtii* mating reaction. In the 1993
experiment, it was also found that \([\text{Ca}^{2+}]_{cyt}\) probably does not increase during the *C. reinhardtii* mating response in response to cAMP signalling, but confirmed the findings of Bloodgood and Levin (1983) that Ca\(^{2+}\) increases in the external media. It had already been shown that mating in *Chlamydomonas* could increase intracellular cAMP concentration tenfold, while addition of external cAMP could induce mating structure formation (Pasquale and Goodenough 1987). The later finding, therefore, that Ca\(^{2+}\) blockers such as LaCl\(_3\) and lidocaine, could block the mating associated increase in cAMP (Goodenough et al. 1993) suggests that Ca\(^{2+}\) plays a role in the initial activation of cAMP signalling, probably via an adenylate cyclase, leading to activation of the mating response (Quarmby 1994).

The flagella of *C. reinhardtii* are associated with mating and undergo deflagellation in response to a variety of stimuli, which relies on Ca\(^{2+}\) (Sanders and Salisbury 1989). Quarmby and Hartzell (1994) discovered that application of benzoate, and the wasp venom mastoparan, could induce deflagellation in a Ca\(^{2+}\)-dependent manner, via two distinct Ca\(^{2+}\)-signalling pathways. Acid treatment with benzoate was found to rely on influx of external Ca\(^{2+}\) in order to trigger deflagellation, which did not occur in the deflagellation-deficient *C. reinhardtii* mutant *adf*-1. Treatment with mastoparan, however, was sufficient to trigger deflagellation in both wild type *Chlamydomonas* as well as the *adf*-1 mutant, while external Ca\(^{2+}\) influx was found to be a response to, rather than a cause of, deflagellation in response to mastoparan, which was instead more likely triggered by release of Ca\(^{2+}\) from internal stores (Quarmby and Hartzell 1994). Other experiments showed that deflagellation in response to both acid shock and mastoparan was dependent on rapid accumulation of IP\(_3\), which occurred between 2-3 seconds after addition of benzoate and preceded the deflagellation event (Quarmby et al. 1992, Yueh and Crain 1993). Much more recently, precise Ca\(^{2+}\) elevations have been observed in *C. reinhardtii* cells in response to CaCl\(_2\) and benzoate treatment, both of which elicited cytosolic Ca\(^{2+}\) increases associated with deflagellation (Wheeler et al. 2008). Taken
together, these results suggest that Ca\textsuperscript{2+} signalling represents a vital step in the deflagellation response of *C. reinhardtii*.

Ca\textsuperscript{2+} signalling has also been shown to play an important role in regulating interflagellar transport (IFT) of accumulated IFT particles during gliding motility of *C. reinhardtii* cells (Collingridge et al. 2013). *Chlamydomonas* cells are able to move through a soil, or water-based medium using their flagella to pull themselves along. Alternatively, *C. reinhardtii* cells have also been observed “gliding” along glass slides, with flagella in contact with the glass surface which then allows cells to move with one lead flagellum pulling the cell along followed by the trailing flagellum then pushing in the same direction. It was shown that Ca\textsuperscript{2+} signalling events occurred within 2 seconds of flagella gliding events, but only in the trailing flagella, which occurred in 84% of cells tested (Collingridge et al. 2013). In the same experiment, it was also shown that Ca\textsuperscript{2+} elevations corresponded with the timing of retrograde IFT clearance of accumulated IFT particles, with 78% of gliding cells tested exhibiting retrograde IFT and each clearance associating with a Ca\textsuperscript{2+} elevation in trailing flagella. The requirement of retrograde IFT for Ca\textsuperscript{2+} was confirmed by performing experiments in a buffer lacking Ca\textsuperscript{2+}, which led to a vastly increased accumulation of IFT particles, while gliding motility was completely inhibited after 5 minutes of treatment without external Ca\textsuperscript{2+} (Collingridge et al. 2013).

A very recent and interesting finding is that the *C. reinhardtii* genome contains 14 homologues of plant CDPKs (Liang and Pan 2013), which have multiple roles in Ca\textsuperscript{2+}-dependent signalling of various responses in higher plants, including the phosphorylation of proteins during signal transduction in response to stimulus-induced intracellular Ca\textsuperscript{2+} increases (Schulz et al. 2013). Investigation into CDPK3, which localises to the *C. reinhardtii* flagella, provides evidence of a role for these proteins in mediating Ca\textsuperscript{2+}-dependent responses in green algae, as well as in higher plants, as RNAi knockdown of *CDPK3* was shown to prevent the ability of *C. reinhardtii* cells to regenerate flagella in the absence of extracellular Ca\textsuperscript{2+} (Liang and Pan 2013). It will be interesting in the future to
see whether, as in higher plants, CDPKs show a similarly broad role in the decoding and relaying of stimulus-induced Ca\(^{2+}\)-dependent stress responses and acclimation.

**Ca\(^{2+}\) channels in *C. reinhardtii*:**

Despite the identification of various Ca\(^{2+}\) channels in the *Chlamydomonas* genome (Verret et al. 2010), there is a relative dearth of molecular characterisation of these proteins in the literature compared with animal and plant models. Of the nine VDCCs identified in the *Chlamydomonas* genome, only one has been defined. Analysis of the *Chlamydomonas ppr2* mutant, which displays a reduced photophobic response due to the inability to produce a flagellar Ca\(^{2+}\) current (Matsuda et al. 1998), revealed that there was a mutation in CAV2 (Fujiu et al. 2009). RNAi knockdown of CAV2 was also shown to cause a defective photophobic response, as only 22% of cells expressing CAV2 at 9% of WT expression showed a response to light shock (Fujiu et al. 2009). CAV2 was found to be homologous to the α1 subunit of vertebrate VDCCs and may represent an ancestral Ca2+ transport protein (Fujiu et al. 2009). CAV2 was also found to localise toward the tip of the flagella in *Chlamydomonas* (Fujiu et al. 2009). Further experiments using expression systems are required in order to properly characterise the role of CAV2 in Ca2+ transport, however, these results suggest CAV2 may be required for producing intraflagellar Ca2+ currents that stimulate the photophobic response in *Chlamydomonas*.

Another *Chlamydomonas* import channel that has been partially characterised in the flagella is the mechanosensitive transient receptor potential (TRP) channel PKD2 (Huang et al. 2007). CrPKD2 was of interest because is a homologue of human PKD2, which is implicated in polycystic kidney disease (PKD) and has been linked to cilia function (Huang et al. 2007). It was found that CrPKD2 localised to the plasma membrane of the flagella and showed a four-fold increase in expression during gametogenesis (Huang et al. 2007). Furthermore, RNAi knockdown of CrPKD2 produced lines that
showed up to a 75% decrease in gamete fusion depending on the extent of the reduction of PKD2 compared to WT (Huang et al. 2007). Gametogenesis in *Chlamydomonas* has been linked with Ca\(^{2+}\) dependent signalling mechanisms (Quarmby 1994) therefore CrPKD2 might be involved in Ca\(^{2+}\) signalling of the *Chlamydomonas* mating response. Additionally, the application of cAMP to cells deficient in CrPKD2 rescued the 16-fold reduction in mating efficiency, possibly by bypassing the need for Ca\(^{2+}\) stimulated cAMP release (Huang et al. 2007).

More recently, it has also been observed that several TRP channels undergo upregulation at the genetic level in response to both deflagellation and flagella biogenesis, with *TRP1, TRP11, TRP15* and *TRP16* showing a greater than 2-fold upregulation in response to deflagellation, and *TRP16* showing a 6-fold upregulation in the first 30 minutes of flagella regeneration (Fujiu et al. 2011). It was also postulated that mechanosensitive channels, such as TRP channels, might mediate the Ca\(^{2+}\) influx at the flagella in order to signal gliding motility (Collingridge et al. 2013). TRP11 of *C. reinhardtii* had previously been shown to localise to the flagella at the proximal region, where bending is prohibited (Fujiu et al. 2011). Furthermore, RNAi knockdown of *TRP11* led to inactivation of the *Chlamydomonas* “avoidance” phenotype, which normally allows cells to move temporarily backwards in response to bumping into one another (Fujiu et al. 2011). Interestingly, inhibition of stretch-activated channels, as well as mechanostimulation of cells in the absence of external Ca\(^{2+}\), either greatly reduced or abolished Ca\(^{2+}\) elevations and retrograde IFT, whereas artificially manipulated stimulation mimicking gliding motility led to Ca\(^{2+}\) transients associated with retrograde IFT (Collingdridge et al. 2013). Taken together, the evidence suggests that stretch-activated TRP channels play an important role in mediating Ca\(^{2+}\) dependent responses in *C. reinhardtii*, probably acting as mechanosensitive Ca\(^{2+}\) channels allowing Ca\(^{2+}\) influx to the flagella.
Another type of receptor that may be linked to Ca\textsuperscript{2+}-stimulated responses in \textit{Chlamydomonas} is IP\textsubscript{3}R, one of which has been identified in the flagella (Pazour et al. 2005). IP\textsubscript{3}R transcript levels were found to substantially increase in response to flagellar excision, which suggests the protein functions specifically in the flagella (Pazour et al. 2005). Various experiments on charophyte algae have shown that IP\textsubscript{3} causes release of Ca\textsuperscript{2+} from internal stores (Biskup et al. 1999, Tazawa and Kikuyama 2003, Wacke et al. 2003). The absence of internal stores in flagella suggests that the \textit{Chlamydomonas} IP\textsubscript{3}R homologue is present in the flagellar plasma membrane (Verret et al. 2010). Much earlier experiments showed that IP\textsubscript{3} is involved in the deflagellation response of \textit{Chlamydomonas} to external stress (Quarmby et al. 1992, Yueh and Crain 1993). Taken together, these results suggest IP\textsubscript{3}R, and other potential IP\textsubscript{3}-receptive channels, may form part of various signal transduction pathways in \textit{Chlamydomonas} flagella by importing calcium into the cytosol and triggering deflagellation.

A protein named channelrhodopsin-1 (ChR1), related to bacteriorhodopsin, has been described in \textit{Chlamydomonas} and expression in Xenopus oocytes suggested it was a light-gated proton channel (Nagel et al. 2002). Channelrhodopsin-2 (ChR2) has since been isolated from \textit{Chlamydomonas} and both ChR1 and ChR2 were shown to be gated directly by light after expression in both Xenopus oocytes and mammalian cells (Nagel et al. 2003). Physiological analysis had previously shown that rhodopsin-mediated influx of Ca\textsuperscript{2+} and H\textsuperscript{+} is necessary for the production of two photocurrents in response to flashes of light (Harz and Hegemann 1991). Weak flashes of light stimulated a photocurrent and caused a change in direction of swimming, while high intensity flashes of light stimulated another current in the flagella which caused a stop response (Harz and Hegemann 1991). Application of Ca\textsuperscript{2+} channel antagonists verapamil and pimozide prevented the generation of light-sensitive photocurrents (Harz and Hegemann 1991), which suggests that influx of Ca\textsuperscript{2+} is necessary in signalling phototaxis. Patch pipette probing of the \textit{Chlamydomonas} eyespot also revealed a role for Ca\textsuperscript{2+} in the generation of a photocurrent, while inhibition
of Ca\textsuperscript{2+} with Mg\textsuperscript{2+} significantly reduced the size of the photocurrent and prevented the flagellar stop response-associated current from occurring (Holland et al. 1996).

**Perspectives:**

Although many of the mechanisms which shape Ca\textsuperscript{2+} signals in plants have yet to be elucidated, there is a growing and comprehensive field of knowledge encompassing the range of biotic and abiotic stimuli which cause plants to exhibit specific Ca\textsuperscript{2+} signalling responses. In contrast, while several Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+} signalling components have been identified in green algae, there is a relative dearth of evidence of Ca\textsuperscript{2+} signalling in response to environmental stimuli. As green algae represent a close photosynthetic relative of higher plants, it will be interesting in the future to discern how these organisms respond to external stimuli, and whether the mechanisms shaping the Ca\textsuperscript{2+} signature are highly conserved between them. Considering that, on the whole, the Ca\textsuperscript{2+} channels of algae resemble more closely those of animal cells, while Ca\textsuperscript{2+} efflux proteins appear to be more conserved between plants and algae, it is of great interest to study the Ca\textsuperscript{2+} signalling responses of organisms such as *C. reinhardtii* in order to study the evolution of Ca\textsuperscript{2+} signalling amongst all Eukaryotes.

**Aims and Objectives:**

The ultimate aim of this PhD project was to better characterise the response of *Chlamydomonas reinhardtii* to environmental stresses, and in particular to elucidate the potential role of CAX proteins in maintaining ion homeostasis and in mediating Ca\textsuperscript{2+} signalling responses. To this end, the experimental work presented in this thesis pertains toward the investigation of three aims. Firstly, to elucidate the role of CAX1 and CAX2 in *C. reinhardtii* in maintaining cation homeostasis, secondly, to demonstrate that *C. reinhardtii* cells exhibit Ca\textsuperscript{2+} responses similar to those seen in plants in response to abiotic stimuli, and finally, to investigate the role, if any, that CAX1 and CAX2 play in
modulating Ca\textsuperscript{2+} signals in \textit{Chlamydomonas}. In order to achieve the aims listed, the following experimental objectives were carried out:

- To investigate the role of CAX1 and CAX2 in maintaining ion homeostasis in \textit{C. reinhardtii}, CAX1 and CAX2 knockdowns were generated and screened for using artificial microRNA (amiRNA) knockdown followed by antibiotic selection. Knockdowns were confirmed using quantitative PCR (qPCR) and grown under a variety of stress conditions including the addition of increasing concentrations of CaCl\textsubscript{2}, CdCl\textsubscript{2}, and NaCl to standard media, based on the documented transport capacity of CAX1 for Ca\textsuperscript{2+}, Cd\textsuperscript{2+} and Na\textsuperscript{+} (Pittman et al. 2009).

- In order to measure the effect of knocking down of CAX on the mineral content of \textit{C. reinhardtii} cells, CAX1 knockdowns were grown in standard media, as well as in 10mM CaCl\textsubscript{2}, and metal and nutrient content was analysed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) and compared to that of wild type.

- As Ca\textsuperscript{2+} signals have not been previously observed in \textit{C. reinhardtii} in response to important abiotic stresses, such as salt shock, the Ca\textsuperscript{2+} signalling capacity of \textit{Chlamydomonas} cells was measured using a biolistically loaded Ca\textsuperscript{2+} responsive dye, based on the method generated by Bothwell et al. (2006). \textit{C. reinhardtii} cells were perfused with CaCl\textsubscript{2}, CdCl\textsubscript{2}, and NaCl, and Ca\textsuperscript{2+} signals were measured based on the increased fluorescence of Oregon Green-BAPTA compared with the non-Ca\textsuperscript{2+}-responsive dye Texas Red.

- Despite evidence suggesting a role for CAX proteins in maintaining vacuolar stores of Ca\textsuperscript{2+}, as well as their predicted role as modulators of cytosolic Ca\textsuperscript{2+} transients, CAXs have no directly documented role in shaping of the Ca\textsuperscript{2+} signature. Ca\textsuperscript{2+} signalling responses were therefore analysed in a CAX1
knockdown compared to wild type under increasing degrees of salt stress. Due to the Ca^{2+}/H^{+} antiport capacity of CAXs, as well as the co-regulation between CAXs and pH, the effect on the CAX1 mutant of pH stress was also compared to that of wild type.
Chapter 2 - Methods:

C. reinhardtii strains:

Two C. reinhardtii background strains were used in this project, obtained from the UK Culture Collection of Algae and Protozoa (CCAP), the wild type 137+ (CC-125 or CCAP 11/32C) and the cell wall deficient 11/32CW15+ mutant (cw15). CAX1 knockdowns cax1-kd19 and cax1-kd1 were generated in the 11/32C background using the biolistic approach which is described below, while the CAX1 knockdown cax1-kd91 and the CAX2 knockdowns cax2-kd1 and cax2-kd11 were generated in the cw15 mutant, which was used for ease of transformation using the glass bead method (Kindle et al. 1990). Cax2-kd1 and cax2-kd11 were obtained from Rachel Webster.

Growth of C. reinhardtii:

C. reinhardtii cells were grown in either liquid medium or on solid plates of 1.5% agar (15g.l⁻¹), using standard Tris-acetate-phosphate (TAP) medium (Gorman and Levine 1965) or TAgP medium in which the organic P source ß-glycerophosphate replaced inorganic P. All solutions were made using MilliQ water (Millipore). Liquid stock cultures of C. reinhardtii were maintained in universal tubes or 40ml tissue culture flasks and cultures grown in cabinets (Sanyo Versatile Environmental Chamber) with a 16h light, 8h dark cycle at light intensity of 105±15μmol.s⁻¹.m⁻² and temperature maintained between 21-23°C.

Artificial microRNA (amiRNA) knockdown of CAX1:

The CrCAX1 and CrCAX2 sequences were analysed for miRNA target sites and amiRNA inserts were designed (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl) for transformation into plasmid vector pChlamiRNA3 (Molnar et al. 2009) (Figure 2.1). Vectors were generated based on the method available in the supplementary materials of Molnar et al. (2009). Briefly, forward and reverse amiRNA oligonucleotides were annealed and dephosphorylated, then ligated into vectors (T4 DNA Ligase, New England Biolabs)
after digestion of both the insert and plasmid with SpeI restriction enzyme (Roche) for 1h at 37°C. Ligated plasmids were transformed into competent DH5-α *Escherichia coli*. Cells were then spread onto Ampicillin-selective, 1.5% agar (15g.l⁻¹) LB plates (Ampicillin 50µg.ml⁻¹). Successfully transformed colonies were screened for presence of the correct insert using colony PCR with primers AmiRNAprecFOR and spacerREV (Table 1). Briefly, a cocktail stick was pricked into single bacterial colonies and then added to 10µl PCR mix, made from components of the FastStart TAQ DNA Polymerase dNTP kit (Roche), with 1µl 10X buffer (including MgCl₂), 1µl each of forward and reverse primers (final concentration 10pmol.µl⁻¹), 0.1µl TAQ Polymerase (final concentration 0.05U.µl⁻¹), 0.2µl dNTP (final concentration 0.2mM) and the reaction made up to 10µl with MilliQ H₂O. PCR reactions were performed using the PTC-200 Peltier Thermal Cycler (MJ Research). The following cycling parameters were used to perform the PCR:

94°C 5min

94°C 30sec

55°C 30sec x30

72°C 30sec

72°C 3min

Successfully ligated vectors were sequenced using the My GATC service (GATC Biotech) and aligned with either amiRNA insert sequence using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Vectors containing the correct sequence were extracted from *E. coli* using the Macherey-Nagel Nucleobond®Xtra-Midi kit. Vectors were digested with KpnI (New England Biolabs) for 1h at 37°C in order to linearise them prior to transformation of *C. reinhardtii*.
Figure 2.1: Artificial microRNA knockdown vector and inserts

A The pChlamiRNA3 vector used for introduction of amiRNA knockdown inserts (Molnar et al. 2009).

B Forward and reverse oligonucleotide sequences for amiRNA knockdown of CAX1.

C Forward and reverse oligonucleotide sequences for amiRNA knockdown of CAX2. Bold letters show actual 20bp amiRNA sequence for CAX1/CAX2 knockdown.
Transformation of *C. reinhardtii*:

*C. reinhardtii* cells were transformed using the glass bead method in the cell wall-less *cw15* background (Kindle 1990), and the Biolistic® PDS-1000/He particle delivery approach (Bio-Rad) in the 11/32C background according to the following methods.

Glass bead transformation:

*cw15* cells were grown for two days to yield ~1-2 x 10^6 cells/ml then gently centrifuged at 400g (MSE benchtop centrifuge) and resuspended in 300μl TAP to give ~1 x 10^6 cells/ml. 100μl 20% (w/v) polyethylene glycol (PEG6000) was added to give 5% (w/v) final concentration. 50μl boiled salmon sperm DNA (10mg.ml\(^{-1}\)) and 300mg of acid-washed, oven baked glass beads (0.4-0.5mm) were added along with 2μg of linearised vector. Cells were vigorously vortexed for 15s and plated on TAP-agar (1.5% w/v) + paromomycin (10μg.ml\(^{-1}\)).

Biolistic delivery of DNA:

11/32C cells were grown for two days to yield ~1-2 x 10^6 cells/ml then gently centrifuged at 400g (MSE benchtop centrifuge) and resuspended in 300μl TAP to give ~1 x 10^6 cells/ml. The method described in the Biolistic® PDS-1000/He particle delivery system manual (Bio-Rad) was used in order to perform the transformation experiment.

Selection of *CAX1* knockdowns:

Transformed *C. reinhardtii* were first identified using antibiotic selection by growing cells on TAP + 1.5% Agar (w/v) plates containing 0.02g.l\(^{-1}\) paromomycin, as amiRNA constructs were inserted into cells in the pChlamiRNA3 vector containing the paromomycin resistance gene *aphVIII*. Glass bead transformation yielded only 1 positive *CAX1* knockdown, *cax1-kd91*, out of 14 paromomycin-resistant colonies screened. Biolistic delivery yielded 2 positive *CAX1* knockdowns out of over 20 paromomycin-resistant colonies screened, *cax1-kd19* and *cax1-kd21*. Colony PCR with the AmiRNAprecFOR and spacerREV primers was used to confirm the presence of the
amiRNA construct in cells, as described previously, but instead of pricking, *C. reinhardtii* cells were boiled in 10mM EDTA in order to quickly extract DNA for PCR.

**RNA extraction:**

Cultures of *C. reinhardtii* were centrifuged (MSE benchtop centrifuge) and pellets were resuspended with 1ml TRlzol® Reagent (Ambion®) per 5-10 x 10⁶ cells by pipetting up and down. The mixture was centrifuged at 3000g for 10min (MSE benchtop centrifuge) at 4˚C. Supernatent was transferred to a fresh 15ml Falcon tube and incubated at room temperature for 5min. 0.2ml Chloroform was added per ml of TRlzol used and the tube was vigorously shaken for 15s then incubated at room temperature for 3min. The mixture was then centrifuged at 3000g for 20min at 4˚C to separate the contents into two layers. The aqueous phase was transferred into 1.5ml Eppendorf tubes and RNA precipitated using 500μl isopropanol per 1ml TRlzol originally added. The mixture was left to incubate for 10min at room temperature then centrifuged at 10 000g for 10min (Eppendorf centrifuge 5415R) at 4˚C. Pellets were dried and washed with 75% ethanol using 1ml per 1ml TRlzol originally added. The mixture was vortexed and centrifuged at 6000g for 5min at 4˚C. Supernatent was removed and pellet dried in air prior to resuspension in 50μl MilliQ water.

**Gene expression analysis:**

The primers used for RT-PCR and qRT-PCR analysis of gene expression are listed in Table 2.1. Primers were produced by Eurofins Genomics. *C. reinhardtii* cells were grown to mid-log phase and transferred to growth for 16h in standard TAP or with TAP + 10mM CaCl₂, then harvested for RNA extraction as outlined above. Following RNA extraction, mRNA concentration was calculated using a Nanodrop 1000 Spectrophotometer. cDNA synthesis was performed using the reverse transcriptase SuperScript III (Life Technologies), or BioScript (Bioline). RT-PCR was performed using the BioMix Red 2X mastermix (Bioline) and a 2720 Thermal Cycler (Applied Biosystems). qRT-PCR was
performed using FastStart Universal SYBR Green Master (ROX) (Roche), and experiments were run and analysed using the ABI Prism 7000 Sequence Detection System (Life technologies). The control gene \textit{CBLP1} was used to normalise samples (Table 2.1), and expression of \textit{CAX1}, \textit{CAX2} and \textit{NCX1} was determined using the comparative threshold cycle (CT) method (Schmittgen and Livak 2008), with LinReg used to calculate PCR efficiency (Hårdstedt et al. 2005).
## Table 2.1: Primers for colony PCR, RT-PCR and qRT-PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAX1_F</td>
<td>CTTCAGCAGCTACATCAACCT</td>
</tr>
<tr>
<td>CAX1_R</td>
<td>GTGAACAGGTTCTTGAACAGC</td>
</tr>
<tr>
<td>CAX1_RT_F</td>
<td>CTCGGAGCTGCGAATGGA</td>
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<tr>
<td>CAX1_RT_R</td>
<td>CAACGTTGCCAAAGGTGGC</td>
</tr>
<tr>
<td>CAX2_F</td>
<td>CTTCCCTGTCGCTGGTGCCCT</td>
</tr>
<tr>
<td>CAX2_R</td>
<td>CGCTGCCTCTTGTTCCACAG</td>
</tr>
<tr>
<td>NCX1_F</td>
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</tr>
<tr>
<td>NCX1_R</td>
<td>CAGACGGGAGTAGATCATC</td>
</tr>
<tr>
<td>RACK1_F (CBLP1)</td>
<td>CTTCTGCATGACCCAGAC</td>
</tr>
<tr>
<td>RACK1_R (CBLP1)</td>
<td>CCCACCAGGTGTTCCTTCAG</td>
</tr>
<tr>
<td>amiRNAprecFOR</td>
<td>GGTGTTGGGTCGGTGTTTTTG</td>
</tr>
<tr>
<td>spacerREV</td>
<td>TAGCGCTGATCCCCACCCACCC</td>
</tr>
</tbody>
</table>
Phenotype analysis of *C. reinhardtii* CAX1 and CAX2 knockdown strains:

In order to compare phenotypic differences between wild type and transgenic *C. reinhardtii*, cells were grown under a variety of nutrient stress conditions (CaCl$_2$: 0.34mM (TAP) – 50mM; CdCl$_2$: 0µM - 100µM; CoCl$_2$: 6.8µM (TAP) - 50µM; NaCl: 0.28mM (TAP) – 200mM; Mannitol: 0mM – 400mM; low phosphate (LP): 0.01mM P (as KH$_2$PO$_4$ and K$_2$HPO$_4$) until stationary phase (7-9 days) or mid-log phase (3 days). *C. reinhardtii* stock cultures were grown to stationary phase, then 200µl was added to three replicate universal tubes or 40ml culture flasks containing a volume of 10ml TAP + treatment. Liquid cell cultures were grown under a 16h light, 8h dark cycle at a photon flux of $105\pm15\mu$mol.s$^{-1}$.m$^{-2}$ and a temperature of 21-23ºC (Sanyo Versatile Environmental Chamber). Optical density of cells measured at a wavelength of 680nm was used to calculate differences between transgenic and wild type *C. reinhardtii* using a Helios Aquamate Spectrophotometer (Thermo Spectronic), except where specified cell counts were performed using a Cellometer Auto T4 (Nexcelom Bioscience).

**Cellular elemental analysis:**

10ml of *C. reinhardtii* cells were grown for 7 days in standard TAP medium, or in 10mM CaCl$_2$ TAP medium, and harvested prior to determination of metal content. Metal content was quantified on a per cell basis and the number of cells was calculated by performing cell counts. Cells were first centrifuged at 3000g for 10min, then resuspended and mixed with 10ml of 1mM EDTA for 5min, before further centrifugation and washing with 10ml Milli-Q water. Cells were centrifuged for another 10min at 3000g and pellets were oven dried at 60ºC for 24h then digested in 0.5ml ultrapure nitric acid (67%) at 100ºC for 3h. Samples were diluted with Milli-Q water to 10ml and analysed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Perkin-Elmer Optima 5300) using internal standards.
**Statistical analysis:**

Data obtained from qRT-PCR, cellular elemental analysis and phenotype analysis of CAX1 and CAX2 knockdowns compared to wild-type were analysed for statistical significance using unpaired t-tests. Each knockdown line was compared to the respective wild type at each treatment concentration. Unpaired t-tests were used as the data was non-continuous. Each experiment shows the results of three biological replicates containing separate whole-populations of *C. reinhardtii* cells. qRT-PCR was analysed using three biological replicates, as well as two technical replicates for each genotype and treatment. Cellular elemental analysis and phenotype analysis experiments were performed with three biological replicates for each genotype and treatment.

**Ca\(^{2+}\) Imaging:**

Ca\(^{2+}\) imaging experiments were performed similarly to the procedure described in Collingridge et al. (2013) based on the biolistic delivery protocol described by Bothwell et al. (2006) and Wheeler et al. (2008). Two dyes were used in order to allow ratiometric imaging of Ca\(^{2+}\) in *C. reinhardtii*, the Ca\(^{2+}\)-responsive green dye Oregon Green-BAPTA Dextran (10000 MW) and the Ca\(^{2+}\)-unresponsive reference dye Texas Red Dextran (10000 MW) (Invitrogen). 0.9mg of 0.6µm gold microcarriers (Bio-Rad) were coated with 40µg Oregon Green-BAPTA and 24µg Texas Red, and the mixture spread thinly to dry on plastic microcarrier discs (Bio-Rad). *C. reinhardtii* cells were grown to mid-log phase and gently centrifuged (400g for 10 min), resuspended in the loading buffer (10mM HEPES pH7.4, 20µM K\(^+\) glutamate, 50mM sorbitol), then ~5 x 10\(^6\) cells were spread onto a 0.45µm nitrocellulose filter (Millipore). Dyes were loaded into cells biolistically using the PDS-1000 delivery system and 1100psi rupture discs (Bio-Rad). Cells were resuspended in TAP medium immediately after loading and left to recover for 2 hours before imaging.

Prior to imaging, *C. reinhardtii* cells were resuspended in an imaging buffer containing 5mM HEPES, 1mM KCl, 1mM HCl, 200µM EGTA and either 0, 250, or 500µM
CaCl₂ (yielding final free Ca²⁺ of 0, 51, or 301µM) and the pH was adjusted to 7.4 using N-methyl-D-glucamine (NMDG). Cells were fixed to 35mm glass-bottomed dishes (In Vitro Scientific) which had been treated with 0.01% Poly-L-Lysine. During Ca²⁺ imaging experiments, *C. reinhardtii* cells were perfused firstly with the imaging buffer for 30s, then with buffers containing added CaCl₂, NaCl, or CdCl₂ for 30s, as presented in the results. Perfusion was performed using gravity flow from multiple taps and a suction system removed excess liquid from the 35mm imaging dish.

Epifluorescence microscopy was performed at room temperature using a Nikon Eclipse Ti with a 100x, 1.49 NA oil immersion objective and an EM-CCD camera (Photometrics Evolve). Texas Red was excited using a 561nm laser (Coherent) with an emission filter of 575-625nm, while Oregon Green BAPTA was excited using a 488nm laser (Coherent) with an emission filter of 500-550nm. For Ca²⁺ imaging experiments, both Oregon Green BAPTA and Texas Red were excited simultaneously and a dual-view beam splitter device was used to detect emission. Images were captured using NIS-Elements v3.1 at 300ms per frame for the majority of experiments (images captured at 200ms per frame include Figure 2.6 A and Figure 3.2 A & B). Images were processed by calculating the mean pixel intensity of defined regions of interest within *C. reinhardtii* cells for each time point, which was used to calculate a fluorescence ratio between Oregon Green BAPTA/Texas Red. To account for any baseline drift, the fluorescence ratio was then normalised by dividing with a rolling median (calculated from a rolling window of 40 time points for CaCl₂ and NaCl imaging, 100 time points for CdCl₂ imaging). Increases in the normalised Oregon Green BAPTA/Texas Red fluorescence ratio of >5% were classed as Ca²⁺ elevations.

**pH imaging:**

*C. reinhardtii* cells grown to mid-log phase were loaded with either 5µM 2',7'-Bis-(2-Carboxethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF-AM) or 5µM Spiro[7H-benzo[c]xanthene-7,1'(3H)-isobenzofuran]-ar'-carboxylic acid, 3-(acetyloxy)-10-
(dimethylamino)-3'-oxo, Acetyloxomethyl Ester (SNARF-AM) (Invitrogen) for 30min, washed and fixed to 35mm glass bottomed dishes (In Vitro Scientific) treated with 0.01% Poly-L-lysine. Both dyes are ratiometric, although BCECF requires dual excitation, whilst SNARF is dual emission. BCECF was excited sequentially at 488nm and 458nm using an LSM 510 confocal microscope (Zeiss) with fluorescence emission at 525±25nm. SNARF was excited at 561 nm using a Nikon Eclipse epifluorescent microscope and detected using an EM-CCD camera (Photometrics Evolve) with a beam splitter device to measure fluorescence emission at 580±25 nm and 630±30 nm. Regions of interest enclosing entire the C. reinhardtii cell were used to measure the average fluorescence intensity for each excitation (BCECF) or emission (SNARF) wavelength and a fluorescence ratio was generated.
Chapter 3 - The role of CAX1 and CAX2 in maintaining ion homeostasis in *C. reinhardtii*:

**Introduction:**

Although *CAX1* and *CAX2* of *C. reinhardtii* have been characterised in transformed yeast cells (Pittman et al. 2009, R. Webster and J. Pittman, unpublished), it was important to characterise their role *in alga*, therefore artificial microRNA (amiRNA) was used to knock down either gene, which has been shown on various occasions to provide robust and specific knockdown of genes in *C. reinhardtii* (Zhao et al. 2009, Molnar et al. 2009). *CAX1* knockdowns were produced in two different *C. reinhardtii* wild-type strains, 11/32C, and *cw15*, the latter of which represents a cell wall-less mutant of the 11/32C background which is useful for efficient glass bead transformation of *Chlamydomonas* (Kindle et al. 1990). The expression of *CAX1* and *CAX2* was measured in both wild type strains compared to knockdowns using quantitative PCR. The expression of another putative Ca$^{2+}$ efflux transporter, a possible Na$^+$/Ca$^{2+}$ exchanger (*NCX1*), was also measured. NCXs form one of the five major families of the Ca$^{2+}$/cation exchanger superfamily of proteins, alongside CAXs, which includes NCX1-3 in animals, as well as AtMHX in plants, with the *C. reinhardtii* NCX clustering with animal NCXs (Emery et al. 2012). *NCX1* is interesting to study due to a potential redundancy in function with CAXs, as well as it’s potentially similar substrate capacity, particularly with regard to Na$^+$ transport.

In order to properly characterise the role of *CAX1* and *CAX2* in *C. reinhardtii*, particularly in regard to their reported capacity for Ca$^{2+}$ and Cd$^{2+}$, and uniquely for *CAX1*, Na$^+$ transport (Pittman et al. 2009, R. Webster and J. Pittman, unpublished), *CAX1* and *CAX2* knockdowns were grown in increasing concentrations of CaCl$_2$, CdCl$_2$ and NaCl, and optical density was compared with wild type. Sequence analysis of *CAX1* and *CAX2* suggests that in algae, *CAX1* might localise to a vacuole-type intracellular compartment, with *CAX2* localising to either the chloroplast or mitochondria (Hanikenne et al. 2005).
Considering the vacuolar localisation of CAX1 and CAX2 in transformed yeast cells (Pittman et al. 2009, R. Webster and J. Pittman, unpublished), it was hypothesised that CAX1 and CAX2 would localise to an intracellular compartment similar to the vacuole of plant and yeast cells, or the lysosome of animal cells, such as an acidocalcisome. Acidocalcisomes are acidic, polyphosphate-rich organelles which are found in a diverse range of organisms from bacteria to mammals, and which are important in Ca\(^{2+}\) regulatory mechanisms in parasitic protists (Docampo and Moreno 2011). Therefore, it was hypothesised that knockdown of CAX1 and CAX2 would lead to a disturbance in the capacity of cells to sequester divalent cations, as well as Na\(^+\), and therefore knockdowns would show a reduced tolerance to growth on high concentrations of CaCl\(_2\), CdCl\(_2\) and NaCl. Additionally, due to the effects of knocking out various CAX isoforms in Arabidopsis on mineral accumulation (Connorton et al. 2012), ICP-AES analysis was used in order to compare the cellular metal content of CAX1 knockdowns with that of wild type C. reinhardtii, as well as the amount of S and P, in order to gauge the importance of CAX in maintaining a proper balance of metals and nutrients in Chlamydomonas.
Results:

Knockdown of CAX1 and CAX2 in C. reinhardtii:

Robust knockdown of genes in C. reinhardtii can be achieved efficiently using artificial microRNAs (Zhao et al. 2009, Molnar et al. 2009). Using the amiRNA vector pChlamiRNA3, CAX1 and CAX2 knockdowns were generated in C. reinhardtii in two wild type strains, firstly using the glass bead method and the cell wall-less mutant background strain cw15. CAX2 knockdown cells were generated by a former lab member (Rachel Webster). Two amiRNA target sites were used in order to generate CAX1 and CAX2 knockdowns in C. reinhardtii cells, which were highly specific for either CAX1 or CAX2. CAX1 and CAX2 knockdowns were identified by repeated selection on paromomycin, with the pChlamiRNA3 vector containing the paromomycin resistance gene aphVIII. As glass bead transformation of cw15 only yielded one CAX1 knockdown over two experiments out of 12 paromomycin-resistant lines generated, more C. reinhardtii CAX1 knockdowns were generated in the 11/32C wild type background using the biolistic approach. Although several paromomycin-resistant colonies were identified over two transformation experiments, only 3 out of 18 lines generated were found to contain the CAX1 amiRNA transcript after colony PCR analysis. Overall, three CAX1 knockdown lines were analysed using qPCR, cax1-kd91 (cw15 background) and cax1-kd19 and cax1-kd21 (11/32C background), as well as two CAX2 knockdowns, cax2-kd1 and cax2-kd11 (cw15 background), based on phenotypic observations which are also presented in this chapter.

qRT-PCR analysis of CAX1, CAX2 and NCX1 expression in wild type and knockdowns:

Initially, the effect of adding 10mM CaCl₂ on the expression of CAX1 was examined in 11/32C and cw15 C. reinhardtii. CAX1 expression could be induced by addition of 10mM CaCl₂ to TAP medium and was upregulated 3-fold in 11/32C, although this increase was not observed in cw15 (Figure 3.1 A). However, another experiment suggested that addition of 10mM CaCl₂ also did not cause a statistically significant increase in the
expression of CAX1 in 11/32C, whereas CAX2 expression was reduced 6-fold (Figure 3.2). Although the increase in expression of CAX1 was inconsistent between experiments, the results indicate that CAX1 and CAX2 are expressed in both cw15 and 11/32C C. reinhardtii under control conditions, and each gene is differently affected by the addition of 10mM CaCl$_2$. The expression of NCX1 was also measured in both 11/32C and cw15 C. reinhardtii, which has not been previously shown despite its annotation in the C. reinhardtii genome (Merchant et al. 2007). NCX1 expression was detected by qPCR in both cw15 (Figure 3.1 C) and 11/32C (Figure 3.2 C) under control conditions, while NCX1 expression increased 5-fold in 11/32C after addition of 10mM CaCl$_2$.

The expression of CAX1 and CAX2 was measured in wild type C. reinhardtii and in knockdowns using qPCR and was compared relative to the expression of a control gene, CBLP1. Firstly, CAX1 and CAX2 knockdowns generated in the cw15 background were analysed for both target and off-target effects of amiRNA knockdown. CAX1 expression was downregulated 5-fold in cax1-kd91 as well as 3-fold in cax2-kd1 (Figure 3.1 B), while CAX2 expression showed a similar pattern, with 4-fold downregulation in cax1-kd91 and 5-fold downregulation in cax2-kd1 (Figure 3.1 C). CAX1 mutants generated in the 11/32C wild type background were also analysed for expression of CAX1 and CAX2. CAX1 did not show significant downregulation in in cax1-kd19 and cax1-kd21 in TAP media even though cax1-kd21 showed a 3-fold reduction in expression (Figure 3.2 A). CAX2 expression was downregulated 6-fold in 11/32C upon addition of 10mM CaCl$_2$, while in Cax1kd19 and Cax1-kd21 CAX2 expression was reduced significantly compared to wild type in normal TAP medium and maintained this low expression at 10mM CaCl$_2$ (Figure 3.2 B).

In order to test whether knockdown of CAX1 and CAX2 might affect the expression of other Ca$^{2+}$ transporters, NCX1 expression was measured in CAX1 and CAX2 knockdowns. Although CAX1 and CAX2 were knocked down to a similar extent in both cax1-kd91 and cax2-kd1, only cax2-kd1 showed a 4-fold reduction in NCX1 expression.
under control conditions (Figure 3.1 C). Neither cax1-kd19 nor cax1-kd21 showed significantly modified expression of NCX1 compared to 11/32C under control conditions or after addition of 10mM CaCl₂, although cax1-kd19 showed a 20-fold increase in NCX1 expression in 10mM CaCl₂ compared to the 5-fold increase observed in 11/32C in 10mM CaCl₂ (Figure 3.2 C).
Figure 3.1: Quantitative PCR analysis of CAX1, CAX2, and NCX1 expression relative to RACK1 in wild type, CAX1 and CAX2 knockdown C. reinhardtii

A CAX1 expression in wild types cw15 and 11/32C in TAP media and in TAP media treated with 10mM CaCl₂. Cells were grown to log phase then treated with 10mM CaCl₂ for 16 hours prior to RNA extraction. B CAX1 expression in cw15, cax1-kd91, cax2-kd1 and cax2-kd11 in TAP media. C CAX2 expression in cw15, cax1-kd91, cax2-kd1 and cax2-kd11 in TAP media. D NCX1 expression in cw15, cax1-kd91, cax2-kd1 and cax2-kd11 in TAP media. (*) represents a significant difference of p < 0.05, (**) p < 0.01 (unpaired T-test from three biological replicates and two technical replicates).
Figure 3.2: Quantitative PCR analysis of CAX1, CAX2 and NCX1 expression relative to RACK1 in 11/32C and CAX1 knockdown C. reinhardtii

Expression of A CAX1, B CAX2 and C NCX1 in 11/32C, cax1-kd19 and cax1-kd21 in TAP media and at 10mM CaCl₂.

Cells were grown to log phase and then treated with 10mM ClCl₂ for 16 hours. (*) represents a significant difference of p < 0.05, (**) p < 0.01, (d) represents a significant difference between CAX2 expression in 11/32C at 10mM CaCl₂ (unpaired T-test from three biological replicates and two technical replicates).
Cellular metal concentrations of *C. reinhardtii* CAX1 knockdowns:

CAX proteins have been shown to be of great importance in maintaining proper mineral acquisition and distribution in plants. *Arabidopsis* cax1/cax3 mutants show decreased Ca and Mg accumulation but increased levels of P, Zn and Mn, measured as total metal concentrations in plant tissue (Cheng et al. 2005). These mutants also show altered distribution of various elements between the seed coat and seed embryo of *Arabidopsis*, while vacuolar sequestration of a range of elements was reduced (Punshon et al. 2012). CAX2 overexpression increases Cd, Ca and Mn levels in tobacco, (Hirschi et al. 2000), while CAX1 overexpressing plants show increased Ca, K, Mg, Mn, P, Zn and Fe (Mei et al. 2007). It was therefore relevant to measure the effect of knocking down CAX on the cellular mineral content of *C. reinhardtii*.

To observe the elemental content of CAX1 knockdowns, *cax1-kd91, cax1-kd19* and *cax1-kd21* were compared with the corresponding parent line (*cw15* and 11/32C, respectively) using ICP-AES analysis. The elements measured were Ca, S, Fe, Mn, Cu and Zn in TAP media (Figure 3.3) and Ca, S, P, K, Fe, Mg, Mn, Cu and Zn in TAP supplemented with 10mM CaCl$_2$ (Figure 3.4). There was a similar distribution of elements between *cw15* and 11/32C when grown under control conditions and in TAP + 10mM CaCl$_2$, although Ca content was much greater in 11/32C (Figure 3.3 A, B). In TAP media, *cax1-kd91* showed a significant increase in Ca, S and Fe accumulation compared with *cw15* (Figure 3.3 A, C), as well as a significant decrease in Mn, Cu and Zn accumulation (figure 3.3 C). *Cax1-kd19* and *cax1-kd21* showed significantly increased S and Fe and reduced Cu compared to wild type, while Mn content was increased in *cax1-kd21* (Figure 3.3 A, D). In 10mM CaCl$_2$, *cax1-kd91* showed a significant increase in Ca content compared to *cw15*, alongside a reduction in S, K, Mg and Cu (Figure 3.4 A, C). *cax1-kd19* and *cax1-kd21* showed a significant increase in Mg and Cu content in 10mM CaCl$_2$ compared to 11/32C, while *cax1-kd19* showed greater S and Fe, and *cax1-kd21* showed reduced K content (Figure 3.4 B, D). Overall, CAX1 knockdowns generated in either
11/32C or cw15 showed similar differences in elemental accumulation compared to wild type, with each of cax1-kd91, cax1-kd19 and cax1kd21 showing increased accumulation of S and Fe, and a decreased accumulation of Cu under control conditions. Although less clear when grown in TAP + 10mM CaCl2, a trend toward increased Ca content was observed between cax1-kd91, cax1-kd19 and cax1kd21, while S, Mg, K and Cu content were also perturbed in multiple CAX1 knockdown lines.
Figure 3.3: ICP-AES analysis of elements in wild type and CAX1 knockdown C. reinhardtii grown in standard TAP medium (0.34mM CaCl$_2$)

The content of various elements measured as metal per cell (µg) in cax1-kd91 (A, C), cax1-kd19 and cax1-kd21 (B, D). Cells grown in standard TAP media (0.34mM CaCl$_2$) for seven days. (*) represents a significant difference of p < 0.05, (**) p < 0.01 (the result of unpaired t-tests between wild type and knockdowns using three biological replicates).
Figure 3.4: ICP-AES analysis of elements in wild type and CAX1 knockdown C. reinhardtii grown in high Ca$^{2+}$ (10mM CaCl$_2$)

The content of various elements measured as metal per cell (µg) in cax1-kd91 (A, C), cax1-kd19 and cax1-kd21 (B, D). Cells grown in TAP media plus 10mM CaCl$_2$ for seven days. (*) represents a significant difference of p < 0.05, (**) p < 0.01 (the result of unpaired t-tests between wild type and each knockdown using three biological replicates).
Growth analysis of CAX1 and CAX2 knockdowns:

Disruption of CAX activity can lead to phenotypic and morphological variations in plants, such as symptoms reminiscent of Ca\(^{2+}\) deficiency in CAX1 overexpressing tobacco (Hirschi 1999). CAX1 overexpressing tobacco plants also display hypersensitivity to growth on media deficient in Ca\(^{2+}\) (Mei et al. 2007). Other CAX mutants show differences in growth in response to a variety of other ions. CAX2 overexpressing tobacco plants are more tolerant to Mn\(^{2+}\) (Hirschi et al. 2000), while loss of function of CAX4 in Arabidopsis leads to altered root growth on media containing Cd\(^{2+}\), Mn\(^{2+}\) and auxin (Mei et al. 2009). More recently CAX1 and CAX3 of Arabidopsis have been implicated strongly in signalling the root-shoot phosphate starvation response (Liu et al. 2011). It was therefore of interest to study the effect of a variety of cations and other abiotic stress conditions on C. reinhardtii CAX1 and CAX2 knockdowns.

CAX1 and CAX2 knockdowns did not show any pronounced growth defect when grown in standard TAP media, but did show differences in growth compared to wild type C. reinhardtii when grown under various abiotic stress conditions, including added CaCl\(_2\), CdCl\(_2\), NaCl and mannitol. As CAX1 has been shown to mediate transport of Ca\(^{2+}\), Cd\(^{2+}\) and Na\(^+\) in transformed yeast cells (Pittman et al. 2009) it was appropriate to measure the response of knockdowns to these ions. The response of knockdowns to CoCl\(_2\), mannitol and low phosphate was also measured. Cax1-kd91 showed a greater sensitivity to growth on CaCl\(_2\), CdCl\(_2\) and NaCl compared to cw15 and a cw15-pChlamiRNA3 vector control, as well as an increased tolerance to growth on mannitol (Figure 3.5). cax1-kd91 showed significant reduction in growth when treated with 10mM, 20mM and 50mM CaCl\(_2\) compared with vector control (Figure 3.5 A), while this effect was also observed at 20mM CaCl\(_2\) compared with cw15, as confirmation of the initial phenotype observed (Figure 3.5 B). A similar pattern was observed in cax1-kd91 compared to vector control in response CdCl\(_2\) (Figure 3.5 C), whereby cax1-kd91 showed significantly reduced growth at 10µM and 50µM CdCl\(_2\). cax1-kd91 showed increased tolerance to growth on 200mM and
400mM mannitol compared to cw15 (Figure 3.5 D). Cax1-kd91 was also more sensitive to growth on increasing concentrations of NaCl, showing significantly reduced growth compared to cw15 at 30mM, 50mM, 100mM and 200mM NaCl (Figure 3.5 E). CoCl$_2$ sensitivity was not found to be different between cax1-kd91 and cw15 (Figure 3.5 F).

In the walled *C. reinhardtii* background, 11/32C, cax1-kd19 and cax1-kd21 showed lesser growth in TAP with added CaCl$_2$ and NaCl (Figure 3.6). cax1-kd19 and cax1-kd21 showed significantly reduced growth at 50mM CaCl$_2$ (Figure 3.6 A) and, in a repeat experiment, cax1-kd21 also showed a significant reduction in growth at 10mM CaCl$_2$, while at 50mM CaCl$_2$ both cax1-kd19 and cax1-kd21 showed reduced growth, but this difference was not found to be statistically significant (Figure 3.6 B). 100mM NaCl was also shown to cause a significant reduction in growth of both cax1-kd19 and cax1-kd21 compared to 11/32C (Figure 3.6 C). Absorbance measurements from a later experiment showed cax1-kd19 was more sensitive to growth on 50mM NaCl (Figure 3.6 D), while cell counts of the same experiment showed cax1-kd12 and cax1-kd21 to have reduced growth at 50mM NaCl compared to 11/32C (Figure 3.6 E). Low phosphate treatment strongly reduced the growth of all genotypes and the sensitivity of cax1-kd19 and cax1-kd21 was not significantly different from that of 11/32C (Figure 3.6 F).

*Cax2-kd1* showed better growth compared to wild type on TAP media supplemented with 20mM and 50mM CaCl$_2$, the effect increasing with concentration (Figure 3.7 A). *Cax2-kd1* therefore showed the opposite phenotype of that observed for cax1-kd91 in response to growth on high CaCl$_2$ (Figure 3.7 B). After the data was normalised to compare like-for-like sensitivity between each *C. reinhardtii* line, both cax2-kd1 and cax2-kd11 showed significantly better growth at 50mM and 100mM NaCl compared to cw15, with a larger effect in cax2-kd1 at 100mM NaCl (Figure 3.7 D).
Figure 3.5: Optical density (OD) of CAX1 knockdown cax1-kd91 when grown on CaCl₂, CdCl₂, Mannitol and NaCl compared to cw15 and a cw15-pChlamiRNA3 vector control

(Figure legend presented overleaf).
**Figure 3.5 (legend):**

**A** OD of *cax1-kd91* grown in TAP, 10mM, 20mM and 50mM CaCl₂ for 7 days, compared to vector control. **B** OD of *cax1-kd91* grown in TAP and 20mM CaCl₂ for 7 days compared to wild type *cw15* measured as absorbance at 680nm. **C** OD of *cax1-kd91* on TAP, 10μM, 20μM, 50μM and 100μM CdCl₂ for 7 days compared to vector control shown as normalised absorbance at 680nm. **D** OD of *cax1-kd91* grown in TAP, 50mM, 200mM and 400mM mannitol for 7 days, compared to wild type *cw15* measured as normalised absorbance at 680nm. **E** OD of *cax1-kd91* grown in TAP, 30mM, 50mM, 100mM and 200mM NaCl for 9 days compared to wild type measured as absorbance at 680nm. **F** OD of *cax1-kd91* grown in 10μM, 20μM and 50μM CoCl₂ for 7 days compared to wild type *cw15* measured as absorbance at 680nm. Normalised absorbances were calculated based on the OD of each *C. reinhardtii* line relative to its own OD in TAP media, which was normalised to equal 1. Normalisation was performed when the growth on standard TAP was too dissimilar between each line to compare OD directly. (*) represents a significant difference of p < 0.05, (**) p < 0.01 (from unpaired t-tests between wild type and knockdown at each different concentration using three biological replicates).
Figure 3.6: Optical density (OD) of CAX1 knockdowns cax1-kd19 and cax1-kd21 on CaCl₂, NaCl, and low phosphate, compared to wild type 11/32C

(Figure legend presented overleaf).
Figure 3.6 (legend):

OD of \textit{cax1-kd19} and \textit{cax1-kd21} compared to 11/32C measured as absorbance at 680nm on increasing concentrations of \textbf{A}, \textbf{B} \text{CaCl}_2\textbf{C} \text{NaCl} for 7 days and \textbf{C} \text{NaCl} for 7 days or \textbf{D} \text{NaCl} for 3 days. \textbf{E} Cell counts of \textit{cax1-kd19} and \textit{cax1-kd21} compared to 11/32C after growth in \text{NaCl} for 3 days and \textbf{F} low phosphate (0.01mM P) for 3 days. (*) represents a significant difference of $p < 0.05$, (**) $p < 0.01$ (from unpaired t-tests between wild type and each knockdown at each different treatment concentration using three biological replicates).
Figure 3.7: Increased tolerance to CaCl₂ and NaCl in cax2-kd1 compared with wild type cw15

A Optical density (OD) of cax2-kd1 and cw15 grown in increasing concentrations of CaCl₂ for 7 days, measured as absorbance at 680nm. B The differing effect of 10mM CaCl₂ on the OD of Cax1-kd91 and Cax2-kd1 compared to growth in TAP media. C OD of cax2-kd1 and cw15 after growth in NaCl for 7 days measured as normalised absorbance at 680nm. Normalised values were calculated for each strain relative to their own growth on TAP media, with growth on TAP equal to 1. (*) significant difference of p < 0.05, (**) p < 0.01 (from unpaired t-tests between wild type and knockdowns at each different treatment concentration using three biological replicates).
Discussion:

**Gene expression of Ca\(^{2+}\) transporters in C. reinhardtii:**

The gene showing the greatest up-regulation in response to 10mM CaCl\(_2\) was NCX1, of which expression increased 5-fold in 11/32C and 20-fold in cax1-kd19. As a putative Na\(^+\)/Ca\(^{2+}\) exchanger in C. reinhardtii, NCX1 provides a similarly interesting case to study in terms of its influence in mediating salt stress and its potentially similar transport ability as CAX1. NCX is a mainly plasma membrane localised protein in animal cells which is important in Ca\(^{2+}\) regulatory mechanisms, primarily in Ca\(^{2+}\) efflux (Philipson and Nicoll 2000). NCX expression has been observed in human (Qu et al. 2000) as well as mice and chick heart cells during development (Linask et al. 2001). The use of an NCX inhibitor was also found to affect heart development in a similar manner to using a Ca\(^{2+}\) ionophore, suggesting a role for NCX in modulating Ca\(^{2+}\) levels (Linask et al. 2001). This is the first time that expression of NCX1 has been shown in C. reinhardtii, and its upregulation in response to elevated CaCl\(_2\) suggests that NCX1 will be interesting to study in the future, especially in relation to stresses such as NaCl.

qRT-PCR analysis of cw15 and 11/32C C. reinhardtii showed that CAX1 expression may be induced by the addition of 10mM CaCl\(_2\). This supports RT-PCR analysis of CAX1 expression previously observed in C. reinhardtii, whereby CAX1 was shown to be induced in response to both addition of 10mM CaCl\(_2\) and 50mM NaCl, but not KCl (Pittman et al. 2009). A significant 3-fold increase in CAX1 expression was observed only in the 11/32C C. reinhardtii background, while this was inconsistent between experiments, and two experiments showed that there was no significant increase in expression of CAX1 in either cw15 or 11/32C, though on a number of occasions RT-PCRs (data not shown) have confirmed the findings of Pittman et al. (2009). The difference between qRT-PCR and RT-PCR may be explained by a greater sensitivity of the qRT-PCR, as well as a more strict analysis based on expression of genes relative to a constitutively expressed control. A more likely explanation may be that CAX1 expression
is transiently induced, and increased expression is not always detectable. It seems that CAX1 is relatively highly expressed in normal conditions, and if anything only undergoes a slight transcriptional upregulation upon induction by CaCl2. Perhaps more interestingly, CAX1 expression appears to be downregulated at higher concentrations of both CaCl2 and NaCl (Pittman et al. 2009), which perhaps suggests that, at higher concentrations, other Ca2+ and Na+ transporters may be induced in order to provide stress tolerance.

The 6-fold downregulation of CAX2 in 11/32C C. reinhardtii in response to 10mM provides an interesting comparison to the upregulation observed in CAX1, and suggests that these two highly homologous proteins play different roles in response to Ca2+ stress. This is not an unexpected effect, as multiple CAX isoforms in Arabidopsis thaliana and rice show differential expression throughout various tissues in response to a diverse range of stimuli. Various CAX isoforms have been shown to be differentially regulated in response to abiotic stresses, with CAX3 showing an upregulation in response to NaCl, and CAX2 showing a downregulation in response to Ca2+ starvation (Maathuis et al. 2003). CAX1 expression has been found to be highest in leaves of Arabidopsis, whereas CAX3 expression was stronger in the roots (Cheng et al. 2005), while CAX2 expression was greatest in flowers and vascular tissue, as well as in the apical meristem of young plants (Pittman et al. 2004). Furthermore, CAX1 has also been shown to mediate high capacity transport of Ca2+ in plants, while CAX2 has a much lower capacity for Ca2+ but shows greater specificity for other divalent cations, such as Mn2+ (Pittman and Hirschi 2003). It is likely that, as with plant CAXs, CAX1 and CAX2 of C. reinhardtii play similar but slightly divergent roles in response to ion stresses.

The role of CAX1 in C. reinhardtii:

It was expected that knockdown of CAX1 would lead to ion sensitivities, particularly in response to excess Ca2+, due to a reduced capacity of C. reinhardtii to sequester divalent cations into an intracellular compartment, particularly considering the Ca2+ and Cd2+ transport ability of CAX1 observed in yeast (Pittman et al. 2009). As with Arabidopsis
cax1/cax3 mutants which showed increased sensitivity to CaCl₂ along with reduced Ca²⁺ content (Cheng et al. 2005), it was expected that CAX1 knockdowns might show a similar phenotype. The increased sensitivity of three independent CAX1 knockdowns, cax1-kd91, cax1-kd19, and cax1-kd21, to CaCl₂ suggests that CAX1 of C. reinhardtii plays a similar role in maintaining Ca²⁺ homeostasis as plant CAXs. The increased accumulation of Ca²⁺ in cax1-kd91 compared with cw15 further supports this observation, although it might have been expected that in CAX1 mutants of C. reinhardtii, there would have been lower Ca²⁺ accumulation. It appears, therefore, that CAX function in C. reinhardtii is similar but not parallel to that in higher plants.

The stronger sensitivity phenotype of cax1-kd91 compared to cax1-kd19 and cax1-kd21 is potentially explained due to a stronger knockdown of CAX1 in cax1-kd91, as well as differences between the wild type C. reinhardtii strains used, such as the presence of a complete cell wall in 11/32C but not in cw15. The much increased Ca²⁺ content of cax1-kd91 compared to cw15 than either cax1-kd19 or cax1-kd21 compared to 11/32C could explain the greater sensitivity to CaCl₂ in cax1-kd91. CAX1 overexpression in tobacco caused an increased accumulation of Ca²⁺, but plants exhibited symptoms reminiscent of Ca²⁺ deficiency (Hirschi 1999). It might be that a similar effect is occurring in C. reinhardtii CAX1 knockdowns, or perhaps the increased sensitivity of knockdowns to Ca²⁺ is due to toxicity of excess Ca²⁺ and other ions due to excess sequestration into an intracellular store. The increased accumulation of Mg and Cu in cax1-kd19 and cax1-kd21 after growth on 10mM CaCl₂ could mean that at high CaCl₂ concentrations, increased sequestration of other ions may contribute to the CaCl₂ sensitive phenotype, as Ca content was not significantly different from the wild type in either knockdown. Whereas, in cax1-kd91, at 10mM CaCl₂ uptake of Ca²⁺ was at least double that of the wild type, therefore it is more likely that altered Ca²⁺ sequestration is the cause of the sensitive phenotype. Cu, K and Mg content was significantly lower in cax1-kd91 at 10mM CaCl₂, however, which might concurrently exacerbate the CaCl₂ sensitive phenotype. It might be
expected that CdCl$_2$ might have had a similar effect in cax1-kd91, either due to a decreased ability of cells to sequester CdCl$_2$, or a similarly increased accumulation of Cd as observed with Ca.

**CAX1 is important to maintain a proper balance of nutrients in *C. reinhardtii***:

The observed changes in elemental distribution between *Chlamydomonas* CAX1 knockdowns and wild type algae can be compared to the effect of knocking out various CAX isoforms in *Arabidopsis*. Ca content was much higher than wild type in cax1-kd91, unlike *Arabidopsis* cax1/cax3 mutants which show increased PO$_4^{3-}$, Mn$^{2+}$ and Zn$^{2+}$ content as well as decreased Ca$^{2+}$ and Mg$^{2+}$ in shoots (Cheng et al. 2005), while in control media Mn and Zn content was lower in cax1-kd91. Cax1-kd91 did show a lower accumulation of Mg, similar to *Arabidopsis* cax1/cax3 mutants, when treated with 10mM CaCl$_2$. This was not consistent between CAX1 knockdowns, however, with cax1-kd19 and cax1-kd21 both showing increased Mg content compared with wild type. However, like cax1-kd91, *Arabidopsis* cax1/cax2 mutants do show an increased Ca and Fe content in seeds, which also exhibit an increase in K and Mn (Connorton et al. 2012). Support for a role for CAX1 and CAX2 of *C. reinhardtii* in mediating Mg nutrition stems from analysis of the *Arabidopsis* cax1 mutant, which displays reduced growth on normal levels of Mg$^{2+}$ and can only grow properly on media supplemented with Mg$^{2+}$ (Bradshaw et al. 2005).

The most commonly shared increase in mineral concentration between different *Arabidopsis* CAX mutants was in Fe, K, and P, which occurred in cax2 and cax2/cax3 seeds, while Zn$^{2+}$ also increased in cax3 seeds along with K and P (Connorton et al. 2012). All three CAX1 knockdowns in *C. reinhardtii* showed an increased accumulation of Fe in control conditions, suggesting that differential Fe accumulation is a common phenotype between plant and algal CAXs. Slightly differing from the effects observed in plants, however, was the decrease in K$^+$ observed in cax1-kd91 and cax1-kd21 after treatment with 10mM CaCl$_2$, suggesting different downstream effects of CAX disruption on K accumulation in *Chlamydomonas* compared with *Arabidopsis*. However, the broad
spectrum of minerals with affected uptake in *C. reinhardtii* CAX knockdowns fits well with the disruption observed in various *Arabidopsis* CAX mutants, and overall points towards the global importance of CAXs in maintaining proper nutrition in both plants and *C. reinhardtii*.

Overall, there was a similar pattern of perturbed elemental accumulation in all three CAX1 knockdown lines compared to wild type. These altered elemental accumulation phenotypes suggest that CAX1 of *C. reinhardtii* is important in mediating nutrient acquisition in *C. reinhardtii*, perhaps through mediating unknown signalling mechanisms or due to a downstream effect on other metal transporters, or possibly due to an as-yet unidentified transport capacity of either CAX1 or CAX2 for cations other than Ca^{2+}, Cd^{2+} and Na^{+}. The link between decreased CAX1 and CAX3 expression and the increase in accumulation of phosphate, as well as an increased shoot/root phosphate ratio under phosphate starvation (Liu et al. 2011) supports the possibility that CAX1 and CAX2 of *C. reinhardtii* are involved in maintaining a proper balance of nutrients in a regulatory capacity. In terms of S accumulation, it would be interesting to see whether the changes observed in CAX1 knockdowns are due to regulation of S transport by CAX1 and CAX2, as with phosphate signalling in higher plants. It might also be the case that phenotypes observed in CAX1 knockdowns, such as Ca^{2+} and Na^{+} sensitivity, as well as a differential accumulation of elements such as Ca and K, have an effect on the gene expression of sulphate transporters. Ca^{2+}, Na^{+}, and K^{+} stress, have been shown to differentially modulate sulphate transport genes in higher plants (Maathuis et al. 2003), therefore the differential S content of *C. reinhardtii* CAX1 knockdowns could accompany ion sensitivity due to a lack of CAX activity.

**Altered metal transport in CAX1 knockdowns:**

Due to the ability of CAXs to transport a diverse range of cations with differing specificities, it is not difficult to suppose that a severe disruption in cation homeostasis due to CAX knockdown might then lead to disturbances in the transport and accumulation of
other cations, potentially via differentially regulated metal transporters. It is known that disruption of CAX activity in plants can lead to downregulation of H^+-ATPases, both at the vacuole (Cheng et al. 2003, Pittman et al. 2004) and at the plasma membrane (Zhao et al. 2008). Plasma membrane H^+-ATPases have themselves been shown to regulate a vast number of plant abiotic stresses, including heavy metal stress (Janicka-Russak 2011). It is possible that differential modulation of H^+-ATPases in C. reinhardtii CAX1 knockdowns might then have downstream effects on the transport of a variety of other metals, including Fe and Cu. In cucumber roots, Cd^{2+} and Cu^{2+} were shown to decrease the ATP-hydrolysing and H^+-transport capacity of the plasma membrane (Kabala et al. 2008), while treatment with 100µM CdCl_2 lowered gene expression of the H^+-ATPase CsHA3 mRNA (Janicka-Russak et al. 2008). In higher plants, Fe transport is also linked with altered H^+-ATPase activity, as well as with the concurrent uptake of other metals including Cd, Co and Ni (Morrissey and Guerinot 2009). Furthermore, K deficiency in plants also involves H^+-ATPase activity at the plasma membrane, which is tied with a downstream signal transduction mechanism involving components of the Ca^{2+} signalling cascade such as CBL and CIPK, which regulate K^+ transport at the tonoplast and plasma membrane (Wang and Wu 2013). Knockdown of CAX1 and CAX2 in C. reinhardtii might therefore have downstream effects on metal transport both due to differential regulation of other proteins, such as H^+-ATPases, as well as due to altered Ca^{2+} signalling responses to nutrient acquisition.

The role of CAX2 in C. reinhardtii:
A surprising contrast to the phenotype observed in CAX1 knockdowns is the CaCl_2 and NaCl tolerance of cax2-kd1. This observation in response to CaCl_2 confirms data previously obtained (Rachel Webster, unpublished) from several CAX2 knockdown lines. The contrast in phenotypes is perhaps more strange considering that both CAX1 and CAX2 are knocked down in all CAX1 and CAX2 knockdowns. This suggests that knockdown of either CAX1, or CAX2, has differing downstream effects on Ca^{2+}
homeostasis, including the differential modulation of the other CAX. It would be interesting to observe the effect of knocking down both CAX1 and CAX2 concurrently using amiRNA in order to gauge the extent of the relationship between them. It certainly appears that CAX1 and CAX2 play complementary roles in relation to ion stress in C. reinhardtii. As yet, there is no data confirming the transport properties of CAX2 in C. reinhardtii. Therefore, as with plants, it might be that CAX2 transports a diverse range of cations other than Ca\(^{2+}\), Cd\(^{2+}\) and Na\(^{+}\).

**Possible co-regulation of CAX1 and CAX2:**
The dual knockdown of CAX2 in the CAX1 knockdowns, as well as the knockdown of CAX1 in the CAX2 knockdown cax2-kd1 was initially surprising. A BLAST search using the amiRNA primers for either gene, as well as the analysis performed by the primer software (WeigelWorld), confirm them to be highly specific for CAX1 and CAX2. It is not implausible that CAX1 and CAX2 are jointly regulated, though it might have been expected that knockdown of either might lead to over-expression of the other. For example, CAX3 and CAX4 RNA levels increased four-fold and three-fold respectively in Arabidopsis cax1 mutants, while CAX2 expression was unaffected (Cheng et al. 2003). The overexpression of CAX3 and CAX4, as well as ACA4, in cax1 plants suggests a compensatory mechanism tied to joint regulation of vacuolar Ca\(^{2+}\) transporters in plants (Cheng et al. 2003). The co-downregulation of CAX1 and CAX2 in C. reinhardtii might then suggest shared regulation at the genetic level, which might be tied to the activity of either CAX1 or CAX2. It could be that in C. reinhardtii, knockdown of CAX1 and CAX2 causes a downregulation in activity of a shared regulator, such as an H\(^{+}\)-ATPase, and that this leads to downstream downregulation of either gene. The differential expression of NCX1 in cax2-kd1 but not cax1-kd91 points toward differences between CAX1 and CAX2 knockdowns despite their apparent co-downregulation.

It was interesting to observe that in cax1-kd19 and cax1-kd21, there was not a significant knockdown of CAX1 when grown in normal medium. This result also occurred
after several RT-PCR analyses (data not shown), whereby CAX1 expression could be induced by addition of 10mM CaCl$_2$ in 11/32C but not cax1-kd19 and cax1-kd21. It is difficult to speculate as to the reason for this, except perhaps for the inherent variance in CAX1 expression in control conditions. Similarly, CAX2 was not downregulated compared to wild type at 10mM CaCl$_2$ in cax1-kd19 and cax1-kd21, though this may be more easily explained due to the strong downregulation of CAX2 also observed in 11/32C at 10mM CaCl$_2$. Overall, amiRNA knockdown was not as strong in any of the CAX1 or CAX2 lines as sometimes observed in previous amiRNA knockdown experiments. Several genes including RBCS2 were knocked down in C. reinhardtii and showed a ten-fold or greater downregulation (Zhao et al. 2009), though another experiment showed knockdown levels in other genes to be at similar levels to those seen in cax1-kd91, cax1-kd19, cax1-kd21, and cax2-kd1 (Molnar et al. 2009). Genes such as RBCS2 are constitutively highly expressed, therefore it is not surprising that the extent of knockdown observed is greater than that seen with genes showing lower basal expression, including CAX1 and CAX2.

**CAX1 and CAX2 play diverse roles in the response of C. reinhardtii cells to NaCl stress:**

Considering the inherent complexity in discerning the nature of the CaCl$_2$ sensitive phenotype of CAX1 knockdowns compared with CAX2 knockdowns, it seems unlikely that there exists a linear relationship between a lack of CAX activity and increased sensitivity to NaCl, especially considering the opposite phenotypes observed in response to salt stress. It could perhaps be that a lack of Na$^+$ sequestration in CAX1 knockdowns cax1-kd91, cax1-kd19 and cax1-kd21 causes an increased sensitivity to NaCl. This seems unlikely, however, as both CAX1 and CAX2 are knocked down to a similar extent in either CAX1 or CAX2 knockdowns lines generated with amiRNA, therefore it would be expected that both would experience similar ionic stresses. As with Ca$^{2+}$, therefore, it is plausible that CAX1 and CAX2 play complementary roles in the signalling of salt stress, perhaps
through indirect regulation of other ion transporters or downstream effects on gene expression.

It is possible that knockdown of CAX activity in CAX1 knockdowns has a downstream effect on the activity of other Na\(^+\) transporters, some of which are driven by H\(^+\)-ATPase activity at the plasma membrane or at the membrane of organelles. The knocking out of cax3 in Arabidopsis has been shown to cause increased sensitivity to NaCl, which was also accompanied by a decrease in activity of a plasma membrane H\(^+\)-ATPase (Zhao et al. 2008). Knockouts of cax1 and cax2 in Arabidopsis also exhibit much reduced V-ATPase activity (Cheng et al. 2003, Pittman et al. 2004). V-ATPase activity has been strongly linked with the activity of Na\(^+\)(K\(^+\))/H\(^+\) exchangers (NHX) at the vacuolar membrane in plants (Pittman 2012, Reguera et al. 2014), which have several homologues in C. reinhardtii that localise to different endomembranes, including vacuole-like compartments, as well as to the plasma membrane (Bassil et al. 2012). Indeed, the direct involvement of Arabidopsis CAX1 in the SOS pathway of plants supports this hypothesis, whereby CAX1 is activated by SOS2, which regulates Na\(^+\)/H\(^+\) exchange at the plasma membrane via SOS1 (Cheng et al. 2004). It is therefore possible that a suite of downstream regulatory processes may be disrupted, or differentially regulated, in C. reinhardtii CAX1 and CAX2 knockdowns, including Na\(^+\) transport independent of CAX1, leading to altered NaCl sensitivity. The increased tolerance to mannitol of cax1-kd91 is also an interesting phenotype to note and might further support a role for CAX in signalling of stress responses in C. reinhardtii. As with cax1 mutants of Arabidopsis which exhibit increased freezing tolerance due to upregulation of CBF/DREB1 related genetic elements (Catalá et al. 2003), it is possible that differential regulation of CAX in Chlamydomonas may alter gene expression in response to abiotic stress. It would be interesting to further investigate whether the increased salt tolerance exhibited in CrCAX1-transformed Arabidopsis seedlings (Pittman et al. 2009) is due to the transport of Na\(^+\) by CAX1, or an up-regulation of salt-responsive pathways such as SOS.
Conclusions and future perspectives:

The knockdown of CAX1 and CAX2 of *C. reinhardtii* using amiRNA has revealed an important and perhaps complementary role for CAX1 and CAX2 in maintaining proper ion homeostasis of Ca$^{2+}$ and Na$^+$. The similarly perturbed mineral content of CAX1 knockdowns, particularly Fe and Cu, as observed in higher plant cax mutants supports a global role for CAX in maintaining proper nutrition. It was striking that both CAX1 and CAX2 were knocked down when attempting to produce single knockdowns of either gene, while these knockdowns showed apparently opposite phenotypes. It would be interesting to try and produce single knockdowns of each CAX1 and CAX2 through overexpressing either protein in the relevant amiRNA knockdown line, in order to gain a better understanding of the individual role that CAX1 and CAX2 play in *Chlamydomonas*. Determining the localisation of both CAX1 and CAX2 in *C. reinhardtii* would also be useful to understand their potential role in sequestering cations. Elucidation of the potential downstream effects of knocking down of CAX activity in *C. reinhardtii* could be gleaned from performing next generation sequencing of transcribed mRNAs in response to different stresses in CAX1 and CAX2 knockdowns, in order to gauge their effect, if any, on gene expression, mainly to highlight genes which are greatly up- or downregulated due to CAX knockdown as targets for further exploration. Targeted qPCR analysis of various transporters, including the organellar and plasma membrane H$^+$-ATPases, would shed further light on the potential ability of CAX to regulate a variety of cell processes including nutrient uptake. It would also be interesting to further analyse other Ca$^{2+}$ transporters of *C. reinhardtii*, including Ca$^{2+}$-ATPases and NCX1, and measure how knockdown of these affects abiotic stress responses.
**Chapter 4 - Rapid, transient cytosolic Ca\(^{2+}\) elevations occur in *C. reinhardtii* in response to external stimuli, including NaCl, CaCl\(_2\) and CdCl\(_2\):**

**Introduction:**

Although widely documented in animals and plants in response to a variety of biotic and abiotic stresses, Ca\(^{2+}\) signals have rarely been directly reported as measurable cytosolic Ca\(^{2+}\) transients in green algae. The unicellular green alga *Eremosphaera viridis* has been shown to display Ca\(^{2+}\) increases in response to a number of stresses, including a “light-off” stimulus, as well as addition of strontium (Sr\(^{2+}\)) and caffeine (Bauer et al. 1997). The “light-off” stimulus caused a transient Ca\(^{2+}\) spiking event, which consisted of a single, large cytosolic Ca\(^{2+}\) increase, with the cytosolic Ca\(^{2+}\) concentration diminishing back to baseline levels in just over a minute. Both 1mM Sr\(^{2+}\) and 20mM caffeine induced an oscillating pattern of Ca\(^{2+}\) spiking, which persisted for over ten minutes, with the caffeine-induced Ca\(^{2+}\) spiking pattern showing reversible inhibition upon addition of Ca\(^{2+}\) channel blockers, including GdCl\(_3\) (Bauer et al. 1997). Further probing of the Sr\(^{2+}\)-induced phenotype revealed that Ca\(^{2+}\) oscillations, as is the case in animal cells, rely on repeated influx and release of Ca\(^{2+}\) from intracellular stores, which was shown to be somewhat dependent on Ca\(^{2+}\) pumps and a ryandodine/cyclic ADP receptor in *Eromesphaera* cells (Bauer et al. 1998).

Ca\(^{2+}\) signals have also been measured with good success in *C. reinhardtii* in response to deflagellation-associated stresses, including addition of 20mM CaCl\(_2\), benzoate, and mastoparan (Wheeler et al. 2008), using a novel method of biolistically-loading the Ca\(^{2+}\)-responsive dye Fluo-4-dextran (Bothwell et al. 2006). *Chlamydomonas* cells exposed to 20mM CaCl\(_2\), in particular, showed either an apical Ca\(^{2+}\) signal alone or a whole-cell Ca\(^{2+}\) signal which appeared to spread in a wave-like fashion throughout the cell.
after an initial sharp Ca\(^{2+}\) spiking event at the apex (Wheeler et al. 2008). These Ca\(^{2+}\) signals were rapid, occurring over a period of seconds, rather than minutes, and also coincided with the timing of the CaCl\(_2\)-induced deflagellation event. Out of 89 deflagellation events, including whole-cell Ca\(^{2+}\) signalling events, most required an apically localised Ca\(^{2+}\) elevation in order to trigger deflagellation. External CaCl\(_2\)-induced deflagellation in \textit{C. reinhardtii}, therefore, appears to be in response to an apically localised Ca\(^{2+}\) signalling event (Wheeler et al. 2008).

Although Ca\(^{2+}\) signalling has clearly been demonstrated in green algae in response to a number of stimuli, which can then lead to a downstream response, such as deflagellation, there is a dearth of evidence for Ca\(^{2+}\) signals generated in response to environmentally relevant stimuli, such as salt shock. In order to measure the Ca\(^{2+}\) signalling response of \textit{C. reinhardtii} to biologically relevant stresses, the Ca\(^{2+}\)-responsive dye Oregon Green-BAPTA was biolistically loaded into cells, and the response of cells to addition of external NaCl and CdCl\(_2\) was measured using fluorescence microscopy. \textit{C. reinhardtii} cells were treated with increasing concentrations of NaCl, ranging from 50 – 120mM, as well as increasing concentrations of CdCl\(_2\) from 100µM – 1mM. In order to confirm the use of Oregon Green-BAPTA as a robust dye for measuring cytosolic Ca\(^{2+}\) transients in \textit{C. reinhardtii}, the response of cells to 20mM CaCl\(_2\) was measured, which has already been shown to elicit a robust Ca\(^{2+}\) signalling response (Wheeler et al. 2008). Additionally, Ca\(^{2+}\) signalling was also measured in \textit{C. reinhardtii} cells using a buffer lacking CaCl\(_2\), in order to gauge the necessity of externally-derived Ca\(^{2+}\) in generating Ca\(^{2+}\) signals. Furthermore, as CAXs are proton-coupled, it was interesting to investigate whether Ca\(^{2+}\) spiking in the cytosol was associated with a whole-cell change in pH, the pH-responsive dye SNARF was used in order to measure any changes in cytosolic pH during Ca\(^{2+}\) signalling events in response to NaCl treatment. The data presented here suggests that \textit{C. reinhardtii} cells respond to NaCl and CdCl\(_2\) stress with rapid Ca\(^{2+}\) signalling events, with the Ca\(^{2+}\) signal increasing with increasing magnitude of the applied

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stimulus. Furthermore, NaCl-induced Ca\(^{2+}\) signals in *Chlamydomonas* appear to form at the cell apex, and spread in a wave-like fashion throughout the cytosol, in a single, rapid Ca\(^{2+}\) signalling event which occurs over a period of just a few seconds. These results show, for the first time, that green algae respond to salt and heavy metal shock with rapid, stimulus-and-dose-specific Ca\(^{2+}\) signals, which form as a wave, comparable to the effects seen in animal and plant cells.
Results:

Measuring cytosolic Ca\(^{2+}\) with Oregon Green BAPTA dextran:

In order to measure Ca\(^{2+}\) signals in *C. reinhardtii* in response to a variety of stimuli, it was important to establish a robust method of measuring Ca\(^{2+}\) signalling using the Ca\(^{2+}\) responsive dye Oregon Green BAPTA dextran. As a reference, the non-Ca\(^{2+}\) binding dye Texas Red dextran was used, which does not show increased fluorescence in response to an increase in Ca\(^{2+}\). Ca\(^{2+}\) signalling events were characterised by an increase in green fluorescence associated with the timing of Ca\(^{2+}\) increases observed by measuring the ratio of Oregon Green fluorescence to that of Texas Red. Oregon Green has previously been used to successfully image Ca\(^{2+}\) elevations in *C. reinhardtii* flagella (Collingridge et al. 2013). Oregon Green BAPTA dextran was preferred over Fluo-4-dextran as Oregon Green has a higher fluorescence at lower concentrations of Ca\(^{2+}\), making it easier to identify well loaded cells. Initial investigations using confocal microscopy revealed Oregon Green to be a suitable dye for imaging cytosolic Ca\(^{2+}\), as there was little interference due to chlorophyll autofluorescence, which meant that epifluorescence microscopy could be used to image Ca\(^{2+}\) signals in *C. reinhardtii*. Ca\(^{2+}\) ionophores were not used to test the efficacy of the dye as these have been shown not to work well in *Chlamydomonas* (Braun and Hegemann 1999).

CaCl\(_2\) treatment triggers repetitive Ca\(^{2+}\) increases in *C. reinhardtii*:

It has previously been shown that *C. reinhardtii* cells respond to external Ca\(^{2+}\) by generating repetitive cytosolic Ca\(^{2+}\) increases when measured with the Ca\(^{2+}\) responsive dye Fluo-4-dextran (Wheeler et al. 2008). These Ca\(^{2+}\) increases consisted of rapid spiking at the apex of cells, as well as the cytosol and flagella, which were associated with a deflagellation event at the same time as Ca\(^{2+}\) spiking. To confirm Oregon Green as a suitable dye, *cw15* cells of *C. reinhardtii* were treated firstly with a pH7.4 HEPES-NMDG buffer containing a low background Ca\(^{2+}\) concentration of 50µM CaCl\(_2\) (Figure 4.1 A). Perfusion of cells with the buffer alone did not trigger an increase in [Ca\(^{2+}\)]\(_{cyt}\), as shown by
the flat ratio between Oregon Green and Texas Red (Figure 4.1 A). Out of ten cells
treated, only one showed an increase in Ca$^{2+}$, while all cells exhibited a flat baseline Ca$^{2+}$
fluorescence. However, treatment with 20mM CaCl$_2$ confirmed the phenotype observed
by Wheeler et al. (2008), whereby Oregon Green fluorescence increased in a similar
manner after perfusion with 20mM CaCl$_2$ for 30s (Figure 4.1 B, C). Cells perfused with
20mM CaCl$_2$ for 30s showed a repeated spiking pattern of cytosolic Ca$^{2+}$ increase (Figure
4.1 B). Just under half of cells (16/35) treated with 20mM CaCl$_2$ responded in this
manner, with the majority showing a similar pattern of one or two large increases in
fluorescence ratio above 5% occurring between 5 and 20s after the stimulus was applied,
followed by several (1 – 5) smaller increases over a maximum period of 60s (Figure 4.1
C).
Figure 4.1: Ca\textsuperscript{2+} oscillations in response to 20mM CaCl\textsubscript{2} in C. reinhardtii

A Overlay of the Oregon green/Texas red ratio of cells treated with a buffer containing 50µM CaCl\textsubscript{2} (n=10). B Example trace from a wild type cw15 cell treated with 20mM CaCl\textsubscript{2} for 30s. Pink bar represents the period of stress application. C Average trace of all cw15 cells responding to treatment with 20mM CaCl\textsubscript{2}. Pink bar represents the period of stress application. (n=16).
**CaCl₂ signals appear to form a wave, initially generated at the apex, then spreading throughout the cytosol:**

In order to better characterise the Ca²⁺ signalling response to addition of 20mM CaCl₂, videos and images were analysed in order to visualise changes in cytosolic Ca²⁺ over time (Figure 4.2) using imageJ. This allowed detailed characterisation of the spatiotemporal properties of the 20mM CaCl₂-induced Ca²⁺ elevations. An example trace (Figure 4.2 A) shows the baseline ratio of Oregon Green / Texas Red along with spiking events, which associated with an increase in Oregon Green, but not Texas Red, fluorescence (Figure 4.2 B, C, D, E). In this typical example of a 20mM Ca²⁺ signalling response, there is a clear increase in the ratio of Oregon Green / Texas Red fluorescence during spiking events (Figure 4.2 C, E), compared to the baseline (Figure 4.2 B, D). Similar to that observed in previous experiments, these Ca²⁺ increases were rapid, lasting less than 3s. Further probing of the spatiotemporal properties of the 20mM CaCl₂ response revealed that the initial Ca²⁺ increase localised to the apex of the cell, which then appeared to spread as a wave throughout the cell from the apex (Figure 4.2 G, H).
Figure 4.2: A representative Ca$^{2+}$ wave in a C. reinhardtii cell under 20mM CaCl$_2$ stress

(Figure legend presented overleaf).
Figure 4.2 (legend):

A Oregon Green / Texas Red ratio of a cw15 cell treated with 20mM CaCl$_2$ for 30s. B, C, D, E False-colour images of the cw15 cell as snapshots taken at 30s, at the height of the Ca$^{2+}$ spiking event, directly after Oregon Green / Texas Red ratio had diminished back to baseline levels, and at the peak of a second, smaller Ca$^{2+}$ increase, respectively. F Oregon Green fluorescence of the cw15 cell. The white arrow indicates the location of the cell apex. G Images of the cw15 cell taken as slices from the cell apex to the posterior and presented side-by-side, representing the changes in cytosolic Ca$^{2+}$ from the apex to the posterior of the cell over the duration of the experiment (0 ms – 105 000 ms). White arrow depicts the beginning of the apically-derived Ca$^{2+}$ increase in response to 20mM CaCl$_2$. Slices representative of frames derived from images of Oregon Green fluorescence taken at an exposure of 300ms. H 20 frames representing images acquired at 300ms exposure before, during and after the 20mM CaCl$_2$-induced Ca$^{2+}$ wave, spanning a window of 3s. The white arrow depicts the initial apical increase in Ca$^{2+}$, which then spreads throughout the cell as a wave in less than 1s. G/H For the benefit of clarity, false colour images represent Oregon Green fluorescence / rolling median Oregon Green fluorescence.
Rapid Ca\(^{2+}\) signalling in *C. reinhardtii* occurs in response to NaCl stress, and increases with the magnitude of the stress applied:

Once a robust method for measuring Ca\(^{2+}\) increases based on Oregon Green fluorescence was developed, the response of *C. reinhardtii* cells to environmentally relevant levels of NaCl stress was measured. Firstly, a control buffer containing 50µM CaCl\(_2\) was used. A number of experiments were performed measuring the response of cells to 50mM NaCl, but although Ca\(^{2+}\) signals were observed in several cells, it was difficult to maintain a robust and replicable phenotype, possibly because the concentration of external Ca\(^{2+}\) available to cells was not sufficient to elicit a reproducible Ca\(^{2+}\) signalling response. Therefore, the amount of CaCl\(_2\) in the buffer was increased to 300µM CaCl\(_2\) by adjusting the amount of EGTA added to the solution, which is similar to the concentration of 340µM used in standard TAP medium.

When treated with 50, 70, 90 and 120mM NaCl for 30s, *C. reinhardtii* cells showed a single, rapid Ca\(^{2+}\) increase which increased with increasing magnitude of the stress applied (Figure 4.3 E). 50mM NaCl stress caused, on average, an 8% increase in the ratio of Oregon green fluorescence over that of Texas red (Figure 4.3 F). At this concentration of NaCl, just over half of cells showed a Ca\(^{2+}\) spike, typically 10-20s after the stimulus (Figure 4.3 A, G, H). Addition of 70mM NaCl triggered a more robust Ca\(^{2+}\) increase, whereby 60% of treated cells showed a Ca\(^{2+}\) spike, typically 7s after the stimulus (Figure 4.3 B, G, H). The average maximum amplitude of the 70mM NaCl induced Ca\(^{2+}\) spike was similar to that of 50mM, showing, with a 9% increase in the ratio of Oregon Green fluorescence compared to Texas Red (Figure 4.3 F).

At 90mM NaCl 75% of cells showed a Ca\(^{2+}\) spike, which typically occurred 5s after the stimulus (Figure 4.3 C, G). The average maximum amplitude of the 90mM NaCl induced Ca\(^{2+}\) spike was a 10% increase in Oregon green fluorescence compared to Texas red (Figure 4.3 F), while several cells exhibited higher ratios of Oregon Green / Texas Red than was observed in any of the cells treated with 50 or 70mM NaCl (Figure 4.3 C).
Treatment of *C. reinhardtii* cells with 120mM NaCl presented the most robust and reproducible Ca$^{2+}$ signalling phenotype, with over 80% of cells responding to the NaCl treatment, typically 5s after the stimulus (Figure 4.3 D, G, H). Several cells showed a 25% increase in the ratio of Oregon green fluorescence compared to Texas red, which was higher than observed in response to any of the other NaCl treatments (Figure 4.3 D), with an average maximum amplitude of 16% (Figure 4 F).
Figure 4.3: NaCl-induced cytosolic Ca\textsuperscript{2+} signalling in *C. reinhardtii*  

(Figure legend presented overleaf).
Figure 4.3 (legend):

A Overlay of the response of multiple cw15 C. reinhardtii cells to 50mM NaCl stress (n=14), measured based on increased fluorescence of Oregon green compared to Texas red. Pink bar represents the duration of the applied NaCl stress, which was applied at 30s. B, C, D Overlay of the response of multiple cells to 70 (n=14), 90 (n=10), and 120mM (n=13) NaCl stress, respectively, measured based on increased fluorescence of Oregon green / Texas red. Pink bar highlights duration of NaCl stress. E Average response of cw15 cells treated with 50 – 120mM NaCl measured based on increased fluorescence of Oregon green compared to Texas red. F Average maximum amplitude of the percentage increase in Oregon green fluorescence compared to Texas Red in cw15 cells treated with 50 – 120mM NaCl. G Average time after the start of the experiment of the first Ca^{2+} increase observed in response to 50 -120mM NaCl treatment. H Percentage of cw15 cells showing a Ca^{2+} signal in response to addition of 50 – 120mM NaCl.
NaCl induced cytosolic Ca\(^{2+}\) transients appear to form as an apically-generated wave in response to 120mM NaCl in *C. reinhardtii*:

Further elucidation of the response of *C. reinhardtii* cells to NaCl treatment was obtained by analysing images and videos of NaCl-induced Ca\(^{2+}\) transients in response to 50mM and 120mM NaCl (Figure 4.4, 4.5) using imageJ, in order to provide detailed information on the spatial characteristics of NaCl-induced Ca\(^{2+}\) spikes. Cell traces representative of the Ca\(^{2+}\) response to 50mM NaCl (Figure 4.4 A) and 120mM (Figure 4.5 A) are presented. Both the increase in Oregon green fluorescence and the ratio of Oregon green / Texas red are noticeably much greater in cells treated with 120mM NaCl (Figure 4.5 B, C, D) compared with those treated with 50mM NaCl (Figure 4.4 B, C, D). Cells treated with 50mM NaCl exhibited an apparently shorter Ca\(^{2+}\) increase than those treated with 120mM NaCl (Figure 4.4 F, G, Figure 4.5 F, G). 120mM NaCl stress was indicated by an initial apex-localised Ca\(^{2+}\) increase, which then spread through the cell in a wave-like fashion (Figure 4.5 G).
Figure 4.4: Ca\textsuperscript{2+} increase in response to 50mM NaCl stress in *C. reinhardtii*

(Figure legend presented overleaf).
Figure 4.4 (legend):

A Oregon Green / Texas Red ratio of a cw15 cell treated with 50mM NaCl for 30s. B, C, D False-colour images of the cw15 cell as snapshots taken at 30s, at the height of the Ca$^{2+}$ spiking event, and directly after Oregon Green / Texas Red ratio had diminished back to baseline levels, respectively. E Oregon Green fluorescence of the cw15 cell. F Images of the cw15 cell taken as slices through the cell which have been placed side-by-side in order to represent the 50mM NaCl-induced cytosolic Ca$^{2+}$ increase over the duration of the experiment (0 ms – 66 000 ms). White arrow indicates the NaCl-induced Ca$^{2+}$ transient. Slices representative of frames derived from images taken at an exposure of 300ms. The repetitive apical increases which occur 6 times are artefacts due to the activity of the contractile vacuole. G 20 frames representing images acquired at 300ms exposure before, during and after the 50mM NaCl-induced Ca$^{2+}$ increase, spanning a period of 3s. White arrow depicts the increase in cytosolic Ca$^{2+}$. F/G For the benefit of clarity, false colour images represent Oregon Green fluorescence / rolling median Oregon Green fluorescence.
Figure 4.5: Ca\textsuperscript{2+} wave in *C. reinhardtii* in response to 120mM NaCl stress

(Figure legend presented overleaf).
**Figure 4.5 (legend):**

A Oregon Green / Texas Red ratio of a *cw15* cell treated with 120mM NaCl for 30s. **B, C, D** False-colour images of the *cw15* cell as snapshots taken at 30s, at the height of the Ca\(^{2+}\) spiking event, and directly after Oregon Green / Texas Red ratio had diminished back to baseline levels, respectively. **E** Oregon Green fluorescence of the *cw15* cell. The white arrow indicates the location of the cell apex. **F** Images of the *cw15* cell taken as slices from the cell apex to the posterior and presented side-by-side, representing the changes in cytosolic Ca\(^{2+}\) from the apex to the posterior of the cell over the duration of the experiment (0 ms – 66 000 ms). Slices representative of frames derived from images taken at an exposure of 300ms. **G** 20 frames representing images acquired at 300ms exposure before, during and after the 120mM NaCl-induced Ca\(^{2+}\) wave, spanning a period of 3s. The white arrow depicts the initial apical increase in Ca\(^{2+}\), which then spreads throughout the cell as a wave in less than 1s. **F/G** For the benefit of clarity, false colour images represent Oregon Green fluorescence / rolling median Oregon Green fluorescence.
NaCl-induced Ca\(^{2+}\) increases in *C. reinhardtii* are reproducible and require influx of Ca\(^{2+}\) from external milieu:

In order to confirm that *C. reinhardtii* cells show a response to NaCl, another wild type strain, 11/32C, was tested. Due to the presence of a complete cell wall, it was difficult to properly image 11/32C cells, which tended to stick to poly-lysine plates via their flagella, meaning that under perfusion with flowing liquid the cells would wobble quite significantly, while others would simply disappear part of the way through the signalling reaction. It was also found that a much higher NaCl stress was necessary to induce a Ca\(^{2+}\) response, perhaps due to the presence of a more robust cell wall, but also perhaps due to the fact that in order to find a well loaded, well stuck, cell, many more 11/32C cells were loaded onto imaging plates than was necessary with *cw15* cells. However, 11/32C cells, when treated with 300mM NaCl, did exhibit a similar increase in Oregon Green / Texas Red fluorescence as observed in *cw15* in response to 90-120mM NaCl stress (Figure 4.6 A). This increase was observable after several repeats, and was of a similar magnitude to that observed in *cw15* cells.

A serendipitous error led to the measurement of Oregon green fluorescence using a dye which did not contain Ca\(^{2+}\)-binding BAPTA in *cw15* cells treated with 120mM NaCl (Figure 4.6 B). This acted as good confirmation that the Ca\(^{2+}\) transients observed using Oregon Green were real, as three cells tested showed a completely flat baseline, absent of any Oregon Green / Texas Red ratio increase of greater than 1%. *Cw15* cells were also treated with 120mM NaCl in the presence of an NMDG-EDTA buffer containing no added CaCl\(_2\). It appears that external Ca\(^{2+}\) is therefore necessary to stimulate NaCl-induced cytosolic Ca\(^{2+}\) increases, as the 8 cells presented (Figure 4.6 C) showed no increase in Oregon Green / Texas Red fluorescence at 37s, while at least 50% of these cells would have been expected to show a signal based on the NaCl treatment.
Figure 4.6: Confirmation of Ca\textsuperscript{2+} signalling phenotype of *C. reinhardtii* in response to NaCl, and the requirement of external Ca\textsuperscript{2+} in order to generate cytosolic Ca\textsuperscript{2+} increases

A  Ca\textsuperscript{2+} signalling in wild type 11/32C *C. reinhardtii* in response to 300mM NaCl stress applied for 30s (pink bar). Blue and red line indicate trace of two separate cells. B Trace of 3 wild type cw15 cells treated with 120mM NaCl for 30s (pink bar) using a non-BAPTA bound Oregon Green dye, which does not bind Ca\textsuperscript{2+}. C Trace of 8 wild type cw15 cells treated with 120mM NaCl for 30s (pink bar) using a buffer lacking CaCl\textsubscript{2}.
No observable change in cytosolic pH in C. reinhardtii cells treated with 120mM NaCl:

In order to gauge whether NaCl stress-induced Ca\(^{2+}\) signalling is associated with a change in pH, \textit{cw15} cells were loaded with the pH indicator SNARF and treated with 120mM NaCl in the same manner as with Ca\(^{2+}\) imaging experiments. SNARF emission was measured at two wavelengths (580nm and 630nm) and the ratio between the two was used to indicate any change in cytosolic pH (Figure 4.7). 120mM NaCl was used due to the robust nature of the phenotype and timing of the Ca\(^{2+}\) response, so that in the absence of any concurrent Ca\(^{2+}\) imaging, it could be expected that at least 50% of cells treated would exhibit a measurable response, if there was one to be found. The functionality of the SNARF indicator was initially tested via NH\(_3\)Cl-induced alkalinisation in \textit{cw15} cells, all of which exhibited a large change in pH (data not shown). None of the cells tested (n=12) showed any alteration in the SNARF ratio over the same time period used to image the Ca\(^{2+}\) signalling response of cells to 120mM (Figure 4.7 A), which was confirmed by producing an average trace (Figure 4.7 B).
Figure 4.7: Measurement of cytosolic pH in response to 120mM NaCl treatment in *C. reinhardtii*

A Combined trace showing the ΔpH of 9 *cw15* cells treated with 120mM NaCl for 30s (pink bar), measured using the pH-responsive indicator SNARF. B Average ΔpH of 9 *cw15* cells treated with 120mM NaCl.
**CdCl₂ stress induces a prolonged cytosolic Ca²⁺ increase in C. reinhardtii:**

Cd²⁺ is an important metal stimulus to study due to its toxicity and presence in agricultural soils and industrial wasteland. In *cw15* *C. reinhardtii* cells, concentrations up to 200µM did not elicit a change in cytosolic Ca²⁺ concentration. When treated with 1mM CdCl₂ for 5, or 2s, *C. reinhardtii* cells showed a 15-20s increase of 5-9% in the Oregon Green / Texas Red ratio, followed by a period of Ca²⁺ oscillation (Figure 4.8) which continued in some cells for over 30min (Figure 4.8 A).
Figure 4.8: Ca\(^{2+}\) signalling in response to CdCl\(_2\) in *C. reinhardtii*

**A** Representative trace of one wild type *cw15* *C. reinhardtii* cell treated with 1mM CdCl\(_2\) for 2s (pink bar). **B** Ca\(^{2+}\) signalling in 6 *cw15* cells treated with 1mM CdCl\(_2\) for 5s (n=1) and 2s (n=5) (pink bar). **C** Average trace of 1mM CdCl\(_2\) Ca\(^{2+}\) signalling events from 6 *cw15* cells.
Discussion:

Rapid, stimulus-specific Ca$^{2+}$ signals are generated by *C. reinhardtii* in response to CaCl$_2$, CdCl$_2$ and NaCl stress:

The data presented show that *C. reinhardtii* cells display Ca$^{2+}$ elevations in response to different abiotic stimuli in a stress-specific manner. CaCl$_2$ and NaCl elicited rapid signalling in *cw15* cells treated for 30s, which occurred as single “spikes” in response to 50-120mM NaCl, and repeated Ca$^{2+}$ increases in response to 20mM CaCl$_2$. On the other hand, CdCl$_2$ elicited a single, prolonged increase in Ca$^{2+}$ which lasted up to 20 seconds, while Ca$^{2+}$ continued to oscillate in the cytosol for periods of over 30 minutes. Although 20mM CaCl$_2$ has previously been shown to induce cytosolic Ca$^{2+}$ oscillations in *C. reinhardtii* (Wheeler et al. 2008), this is the first time Ca$^{2+}$ signals have been reported in green algae in response to salt and heavy metal stress.

The rapidly occurring Ca$^{2+}$ signal in *C. reinhardtii* in response to CdCl$_2$ presented here represents a faster response than has been observed in plant and animal cells. There are not many examples of Ca$^{2+}$ increases in plants in response to Cd$^{2+}$ stress, though rice roots labelled with Oregon Green have been shown to accumulate Ca$^{2+}$ after treatment with 400µM CdCl$_2$ (Yeh et al. 2007). Measurement of Ca$^{2+}$ transients from single cells have been gathered using tobacco suspension cells transformed with aequorin, in which CdCl$_2$ caused a prolonged increase in cytosolic Ca$^{2+}$ lasting several minutes, which increased dependent on dosage to a maximum Ca$^{2+}$ elevation at 1mM CdCl$_2$ (Garnier et al. 2006). A similar effect has been observed in human skin fibroblasts, which showed a large, dose-dependent increase in cytosolic Ca$^{2+}$ in response to 1µM CdCl$_2$ that lasted 1min (Smith et al. 1989). This effect was also observed in human renal epithelial cells, which showed a similar dose-dependent spike of cytosolic Ca$^{2+}$ in response to 0.05 – 1mM CdCl$_2$, though in this experiment, as with tobacco cells (Garnier et al. 2006), the cytosolic Ca$^{2+}$ concentration remained high for several minutes (Faurskov et al. 2002). CdCl$_2$ treatment also increased the production of inositol phosphates, such
as IP₃, in several experiments using animal cells (Smith et al. 1989, Yamagami et al. 1998, Faurskov et al. 2002), suggesting that the cytosolic Ca²⁺ release may be generated via IP₃-sensitive intracellular stores. It would be interesting to measure whether, as with animal cells, C. reinhardtii cells increase the production of IP₃ in response to CdCl₂ stress.

The data presented here shows, for the first time, that C. reinhardtii cells respond to NaCl stress with rapid, dose-dependent cytosolic Ca²⁺ signals, which occur more quickly and with greater magnitude as stress concentration is increased from 50mM up to 120mM NaCl. The Ca²⁺ spikes observed in Chlamydomonas are similar to those previously observed in aequorin-expressing Arabidopsis roots exposed to NaCl for 5s, in which 14-fold cytosolic Ca²⁺ increases were observed in the epidermis, elongation zone, and endodermis, as well as an 8-fold increase in the pericycle (Kiegle et al. 2000). The Arabidopsis root NaCl Ca²⁺ signal included a characteristic rapid spiking event, followed by a gradual reduction in Ca²⁺ concentration lasting for just over 20s, while in the endodermis and pericycle there was a more sustained Ca²⁺ elevation for up to 120s after the initial spiking event. Although the NaCl-derived Ca²⁺ signals observed in C. reinhardtii and Arabidopsis root cells show a similar pattern in terms of the shape of the Ca²⁺ spike, as well as the timing of the response after the stress was applied, the duration of the cytosolic Ca²⁺ increase was up to ten times greater in Arabidopsis. In particular, the oscillation in cytosolic Ca²⁺ observed in Arabidopsis endodermis and pericycle cells, as well as the more elongated duration of the Ca²⁺ signal (Kiegle et al. 2000), is more reminiscent of the CdCl₂ Ca²⁺ signalling response of C. reinhardtii presented here, although these Ca²⁺ oscillations occurred over a much longer duration.

A particularly striking phenotype of the NaCl-stimulated Ca²⁺ signalling response of C. reinhardtii was the increased magnitude of the response based on the strength of the applied stress. Not only were Ca²⁺ spikes larger, and more rapidly occurring, in cells treated with 120mM NaCl compared with 50mM NaCl, but the percentage of cells showing a Ca²⁺ response also increased from 55% to over 80%. Similar effects have been
previously observed in plant Ca\textsuperscript{2+} signalling experiments. For example, stepwise decreases in temperature have been shown to elicit cytosolic Ca\textsuperscript{2+} spikes of increasing magnitude correlating with a reduction in temperature (Plieth et al. 1999). More relevant to the C. reinhardtii NaCl phenotype presented here, Arabidopsis seedlings also exhibit a larger Ca\textsuperscript{2+} increase in response to 150mM NaCl than 50mM NaCl (Donaldson et al. 2004). It has been postulated that a likely outcome of increasingly strong Ca\textsuperscript{2+} transients in response to higher stress concentrations is that a threshold of cytosolic Ca\textsuperscript{2+} must be reached in order for cells to elicit a response, in terms of both the magnitude and duration of the Ca\textsuperscript{2+} transient (Knight and Knight 2001). Such an effect can be manipulated using artificially-generated Ca\textsuperscript{2+} transients, which were shown to decrease stomatal aperture in Arabidopsis guard cells dependent on the frequency of applied Ca\textsuperscript{2+} oscillations (Allen et al. 2001). It would be interesting to observe whether the greater Ca\textsuperscript{2+} signalling response of C. reinhardtii cells to increasing NaCl stress would lead to a greater response in terms of the gene expression of salt-responsive genetic elements, the activation of Na\textsuperscript{+} transporters, and other downstream processes. It would also be interesting to explore whether, considering the longer lag phase of the 50mM NaCl signal observed in C. reinhardtii compared to that observed under 70 – 120mM NaCl stress, these represent two different signalling mechanisms, especially considering that in C. reinhardtii cells, expression of CAX1 increases at 50mM NaCl, but decreases at 100 and 200mM NaCl, compared to control (Pittman et al. 2009).

\textbf{Ca\textsuperscript{2+} signals in C. reinhardtii appear to start at the apex and form waves through the cytosol, which rely on Ca\textsuperscript{2+} influx from the external milieu:}

As previously observed in C. reinhardtii (Wheeler et al. 2008), cw15 cells appear to respond to 20mM CaCl\textsubscript{2} with apically generated Ca\textsuperscript{2+} transients which spread as a wave throughout the cytosol. These apical and whole-cell Ca\textsuperscript{2+} signalling events were accompanied by deflagellation in stressed cells (Wheeler et al. 2008), therefore it is possible that the Ca\textsuperscript{2+} wave observed in 120mM NaCl-stressed C. reinhardtii cells might
signal a similar deflagellation event in response to high osmotic stress. In higher plants, Ca$^{2+}$ waves have been observed in response to a variety of abiotic stresses and developmental cues, however it has often been difficult to properly characterise waves and oscillations due to the high stress concentrations imposed upon cells in order to measure quantifiable Ca$^{2+}$ increases, meaning that waves are often obscured by larger, transient increases in cytosolic Ca$^{2+}$ concentration (Malho et al. 1998). Ca$^{2+}$ waves have been observed in the moss *Physcomitrella patens* in response to stimulation by UV-A (Tucker et al. 2005), and showed a similar pattern to *C. reinhardtii* cells under CaCl$_2$ and NaCl stress, whereby an initial apical Ca$^{2+}$ elevation was followed by a rapid wave which then spread through the cytosol. Again, however, the CaCl$_2$ and NaCl-triggered Ca$^{2+}$ waves observed in *C. reinhardtii* occurred much more rapidly, spreading throughout the cell and depleting in under 3 seconds. It has recently been suggested that Ca$^{2+}$ waves occur throughout eukaryotes, including *C. reinhardtii*, in response to stretch-activated Ca$^{2+}$ influx, with a rate in *C. reinhardtii* as quick as 200µm/s (Jaffe 2007), which supports the observation presented here.

Considering that, in the absence of external Ca$^{2+}$, no Ca$^{2+}$ spiking was observed in response to NaCl stress, it seems likely that the NaCl-induced spiking in *C. reinhardtii* requires Ca$^{2+}$ influx from the external milieu. This contrasts slightly with the effects observed in *Arabidopsis* under NaCl stress, whereby the NaCl-induced Ca$^{2+}$ spike could only partially be repressed by addition of La$^{3+}$ and EGTA (Knight et al. 1997). In *Arabidopsis*, it has recently been observed that Annexin1 (ANN1), a putative plasma membrane Ca$^{2+}$-permeable channel, is responsible for a Ca$^{2+}$ dependent Ca$^{2+}$ influx in the presence of 220mM NaCl (Laohavisit et al. 2013). Although single copies of Annexin homologues are found in several genera of unicellular green algae, none have yet been identified in the genome of *C. reinhardtii* (Jami et al. 2012). It is therefore likely that Ca$^{2+}$ influx in response to NaCl occurs via a different proteins in *C. reinhardtii*, probably more similar to those of animal cells, such as members of the TRP family (Verret et al. 2010).
Another potential candidate from plants, however, is the recently characterised OSCA1 (Reduced hyperosmolality induced $[\text{Ca}^{2+}]_i$ increase 1) of *Arabidopsis*, which is a newly discovered plasma membrane channel with a potential role in osmosensing (Yuan et al. 2014). *Osca1* mutants showed a reduced $\text{Ca}^{2+}$ increase in response to sorbitol compared to wild type plants, as well as altered responses to sorbitol including much increased stomatal aperture (Yuan et al. 2014). Considering that several homologues to OSCA1 have been identified in *C. reinhardtii* (Yuan et al. 2014) it will be interesting to investigate whether homologous proteins may play a role in initial $\text{Ca}^{2+}$ influx in response to osmotic stresses, including salt stress, in *C. reinhardtii*.

Considering the wave-like pattern of $\text{Ca}^{2+}$ increase through the cytosol, it is likely that $\text{Ca}^{2+}$ release from intracellular stores occurs rapidly downstream of the initial apical $\text{Ca}^{2+}$ signalling event. Very recently, NaCl-induced $\text{Ca}^{2+}$ waves were observed in *Arabidopsis* seedlings, which spread rapidly through cells at a rate of up to 400$\mu$m/s (Choi et al. 2014), a more comparable velocity to that exhibited by *C. reinhardtii* cells under 120mM NaCl stress. Similarly to *C. reinhardtii* cells treated with 120mM NaCl in a 0mM $\text{Ca}^{2+}$ buffer, exogenous treatment of *Arabidopsis* roots with $\text{Ca}^{2+}$ channel blockers inhibited the $\text{Ca}^{2+}$ wave in response to 100mM NaCl (Choi et al. 2014). Furthermore, analysis of *tpc-1* mutants showed disruption in both the generation of a $\text{Ca}^{2+}$ wave and in the expression of NaCl-responsive genetic elements compared to wild type, suggesting a role for *Arabidopsis* TPC1, a tonoplast $\text{Ca}^{2+}$ channel, in propagating the NaCl-stimulated $\text{Ca}^{2+}$ wave (Choi et al. 2014). *C. reinhardtii* cells do not contain a TPC homologue (Verret et al. 2010), therefore intracellular $\text{Ca}^{2+}$ release must occur via a different mechanism, although TPC1 alone is not sufficient to generate the *Arabidopsis* NaCl-induced $\text{Ca}^{2+}$ wave phenotype, and may act more as a mediator of whole-plant $\text{Ca}^{2+}$ waves in response to salt (Choi et al. 2014).

As with plants and animals, *C. reinhardtii* contains a number of IP$_3$-responsive channels, including a homologue of animal IP$_3$Rs, which represent a likely source of
intracellular Ca\(^{2+}\) release (Verret et al. 2010). Other possible candidates for ligand-responsive Ca\(^{2+}\) channels driving intracellular Ca\(^{2+}\) release in *C. reinhardtii* are GLRs and CNGCs, of which there are 1 and 3 in the *Chlamydomonas* genome, respectively (Verret et al. 2010). In plants, it has long been established that IP\(_3\)R can mediate Ca\(^{2+}\) release from vacuoles (Sanders et al. 1995). In *Arabidopsis* seedlings under salt stress, it has also been shown that both external Ca\(^{2+}\) and IP\(_3\) contribute to NaCl-induced Ca\(^{2+}\) transients, with EGTA treatment partially reducing the externally-derived Ca\(^{2+}\) transient, and IP\(_3\) signalling inhibitors reducing the vacuole-associated Ca\(^{2+}\) transient (Knight et al. 1997). It was since shown that increased IP\(_3\) accumulation was exhibited by root tip cells, which was associated with a Ca\(^{2+}\) transient, in response to 250mM NaCl stress (DeWald et al. 2001), although this correlation occurred over minutes, rather than seconds. It will therefore be interesting in the future to measure IP\(_3\) levels in NaCl-stressed *C. reinhardtii*, as well as using IP\(_3\) inhibitors, as well as IP\(_3\)R knockdown and mutant lines, in order to further investigate the contribution of IP\(_3\)-mediated intracellular Ca\(^{2+}\) release in *C. reinhardtii* in the response of cells to NaCl. As well as IP\(_3\), the cyclic nucleotides cAMP and cGMP were able to reduce the level of Na\(^+\) influx in *Arabidopsis* roots, while their application increased salt tolerance and lowered accumulation of Na\(^+\) (Maathuis and Sanders 2001). Furthermore, salt stress has been shown to induce rapid accumulation of cGMP in *Arabidopsis* seedlings, with higher cGMP accumulation observed as the salt stress was increased from 50mM to 150mM (Donaldson et al. 2004). There are, therefore, a number of possible mechanisms by which plants and *C. reinhardtii* might generate intracellular Ca\(^{2+}\) release in response to similar stresses, which will be of interest to fully dissect in the future. It is possible, or more likely probable, a number of these ligands and proteins act concurrently in order to generate a full salt stress response.

**Conclusions:**

The unique Ca\(^{2+}\) signatures exhibited by *C. reinhardtii* to different abiotic stresses presented here are novel, and present the first evidence of Ca\(^{2+}\) signalling in green algae.
in response to environmentally relevant stresses, such as salt and heavy metals. The data presented here only begin to explore Ca\(^{2+}\) signalling in *C. reinhardtii*, and further analysis is necessary in order to understand the precise mechanisms shaping these responses, such as the involvement of ligand-gated Ca\(^{2+}\) channels and cell organelles. However, it appears that calcium waves form a conserved response to abiotic stress throughout eukaryotic organisms. The unique pattern of rapid spiking in *C. reinhardtii* cells represents a divergence from those observed in plant cells, occurring over much shorter period. The presence of varied ion channels in *C. reinhardtii* showing homology to both animal and plant proteins, as well as the similar nature of *C. reinhardtii* Ca\(^{2+}\) signalling to both plants and animals, suggests that *C. reinhardtii* is a useful model to study the evolution of Ca\(^{2+}\) signalling in eukaryotic organisms.
Chapter 5 - Investigating the role of CAX in stress signalling in *C. reinhardtii*:

Introduction:

There are two functions that CAX might play in Ca$^{2+}$ signalling, firstly in directly modulating Ca$^{2+}$ elevations through direct Ca$^{2+}$ sequestration and secondly in maintaining an intracellular store of Ca$^{2+}$ prior to stimulus induced Ca$^{2+}$ release. Due to gene redundancy in higher plants, it has been difficult to gauge the precise role, if any, of CAX transporters in modulating plant Ca$^{2+}$ responses to abiotic stresses. Indirect evidence of a role for CAX in generating cytosolic Ca$^{2+}$ oscillations in response to abiotic stimuli comes from analysis of the responses of *Arabidopsis det3* mutants, in which disruption in V-ATPase activity led to a reduced Ca$^{2+}$ response after stimulation by Ca$^{2+}$ and H$_2$O$_2$, potentially due to reduced CAX activity because of a lack of proton flux at the vacuolar membrane (Allen et al. 2000). Due to their high capacity Ca$^{2+}$ transport ability, it is also likely that in plants CAXs are important in establishing the vacuole as a central store of Ca$^{2+}$ which can then be mobilised via intracellular Ca$^{2+}$ channels (Pittman 2011). However, despite evidence that CAXs may be important in the signal transduction of various abiotic stresses, a direct role for them in modulating Ca$^{2+}$ signals has yet to be demonstrated.

As the genome of *C. reinhardtii* has been shown to contain only two expressed CAX isoforms, as well as substantially fewer Ca$^{2+}$-ATPases than are present in higher plants (Pedersen et al. 2012, Emery et al. 2012), *C. reinhardtii* represents a useful model organism to further elucidate the role of CAX in Ca$^{2+}$ signalling. The results presented in Chapter 4 suggest that *C. reinhardtii* cells respond to increased doses of NaCl stress with a highly reproducible Ca$^{2+}$ signalling response, particularly under 70 -120mM NaCl stress, whereby cells exhibited rapid, single increases in cytosolic Ca$^{2+}$. Due to the Na$^+$ transport capacity of CAX1 (Pittman et al. 2009), we examined whether the altered response to NaCl stress of CAX1 and CAX2 knockdowns was due to a change in capacity of cells to transport Na$^+$, or whether knockdown of CAX might lead to a disrupted Ca$^{2+}$ signalling phenotype. In order to investigate whether knockdown of CAX might affect the ability of
C. reinhardtii cells to form a proper Ca\(^{2+}\) signalling response, the CAX1 knockdown cax1-kd91 was imaged alongside cw15 cells using Oregon Green-BAPTA and Ca\(^{2+}\) signalling in response to 70 and 120mM NaCl shock was measured. Considering the Ca\(^{2+}\)/H\(^{+}\) antiport function of CAXs, as well as evidence for their joint regulation with pH in higher plants (Pittman et al. 2005), the response of the CAX knockdowns to acid shock was also investigated, a stimulus that has previously been shown to elicit cytosolic Ca\(^{2+}\) increases in Chlamydomonas (Wheeler et al. 2008). To this end, the pH-responsive dye BCECF was loaded into cw15, cax1-kd91 and cax2-kd1, and cells were treated with both 10mM and 60mM benzoate, in order to compare the effect of acidification on cytosolic pH between CAX knockdowns and the cw15 background.
Results:

Continuous Ca\(^{2+}\) oscillation and a reduced capacity to respond to NaCl stress in \textit{cax1-kd91}:

It has been shown in Chapter 4 that \textit{C. reinhardtii} cells respond to perfusion with NaCl, which elicits a rapid Ca\(^{2+}\) spike in the cytosol at concentrations ranging from 50 – 120mM. In order to investigate whether Ca\(^{2+}\) signalling might be altered in \textit{CAX1} knockdowns, \textit{C. reinhardtii cw15} cells were first perfused with either 70mM or 120mM NaCl, before \textit{cax1-kd91} cells were then perfused with the same treatments, using the same 300\(\mu\)M CaCl\(_2\), 1mM KCl, HEPES-NMDG buffer at pH 7.4. When treated with 70mM NaCl, \textit{cw15} cells showed a rapid increase in Oregon green fluorescence compared to Texas red around 7s after the NaCl stress was applied in over half of the cells tested (Figure 5.1 A). On the other hand, none of the \textit{cax1-kd91} cells tested exhibited any significant Ca\(^{2+}\) increases in response to 70mM NaCl, except for a large increase in Ca\(^{2+}\) in one cell around 30 seconds after the stimulus was applied, which probably represents a spontaneous Ca\(^{2+}\) elevation unrelated to the stimulus (Figure 5.1 B, E). Similarly, when treated with 120mM NaCl, over half of the \textit{cw15} cells imaged showed a rapid Ca\(^{2+}\) increase around 6-7s after the stress was applied (Figure 5.1 C), which was absent in \textit{cax1-kd91} (Figure 5.1 D, F).

In a repeat experiment performed one year later, \textit{cw15} and \textit{cax1-kd91} cells were again treated with 120mM NaCl in order to confirm whether Ca\(^{2+}\) signalling was entirely absent in \textit{CAX1} knockdowns in response to salt stress. It is interesting and important to note that \textit{cax1-kd91} cells did not have any flagella when imaged the first time (Figure 5.3), whereas both \textit{cw15} and \textit{cax1-kd91} cells were flagellated in the repeat experiment. The knockdown of \textit{CAX1} in \textit{cax1-kd91}, however, was maintained as expression levels of \textit{CAX1} remained low. Similarly to the results of the previous year, over half of \textit{cw15} cells treated with 120mM NaCl responded with a rapid increase in Oregon green fluorescence compared to Texas red, which occurred between 5-7s after the stimulus was applied (Figure 5.2 A). Unlike the previous year, however, 30\% \textit{cax1-kd91} cells did show a Ca\(^{2+}\)
increase in response to 120mM NaCl treatment (Figure 5.2 B), compared to 58% of cw15 cells (Figure 5.2 D). The results from 9 cw15 cells and 11 cax1-kd91 cells show that, on average, the Ca\textsuperscript{2+} response was slightly later, and of a greater magnitude, in cax1-kd91 (Figure 5.2 C), with 5 out of 11 cells responding 7.5s after the stimulus in cax1-kd91 compared with only 2 out of 9 cw15 cells (Figure 5.2 F). Probably due to the increased magnitude of the increase in Oregon green fluorescence in cax1-kd91, 4 out of 11 cells had a Ca\textsuperscript{2+} increase lasting longer than 3 seconds, which only occurred in 1 out of 9 cw15 cells in response to 120mM NaCl (Figure 5.2 E).

Another interesting difference to note between cw15 and cax1-kd91 cells was the baseline noise of the Oregon green / Texas red ratio. Although increases in the ratio of Oregon green fluorescence compared to Texas red greater than 2.5% were observed in cw15 cells irrespective of applying NaCl this only happened in several cells tested (Figure 5.1 A, C, Figure 5.2 A), whereas these fluctuations occurred more frequently in cax1-kd91 cells (Figure 5.1 B, D, Figure 2 B). In two out of the three experiments, cax1-kd91 cells showed repeated Ca\textsuperscript{2+} spiking, with the ratio of Oregon Green / Texas Red increasing by 5% or greater (Figure 5.1 D, Figure 5.2 B), while the baseline noise of the Oregon Green / Texas Red ratio was noticeably lesser in the cw15 background (Figure 5.1 C, Figure 5.2 A).
Figure 5.1: Ca$^{2+}$ signalling in *cw15* and *cax1-kd91* under NaCl stress

(Figure legend presented overleaf).
**Figure 5.1 (Legend):**

**A** Overlay of Ca response traces of multiple *cw15* cells (*n*=14) treated with 70mM NaCl for 30s (pink bar). Traces show an elevation in cytosolic Ca$^{2+}$, represented by an increase in Oregon Green BAPTA fluorescence compared to that of Texas Red. **B** Overlay of multiple *cax1-kd91* cells (*n*=11) treated with 70mM NaCl for 30s (pink bar). **C** Overlay of multiple *cw15* cells (*n*=11) treated with 120mM NaCl for 30s (pink bar). **D** Overlay of multiple *cax1-kd91* cells (*n*=14) treated with 120mM NaCl for 30s (pink bar). **E** Mean trace of all *cw15* (blue) and *cax1-kd91* (red) cells treated with 70mM NaCl. **F** Mean trace of all *cw15* (blue) and *cax1-kd91* (red) cells treated with 120mM NaCl.
Figure 5.2: Ca\(^{2+}\) signalling in *cw15* and *cax1-kd91* under NaCl stress (series 2)

(Figure legend presented overleaf).
**Figure 5.2 (legend):**

A Overlay of multiple *cw15* cells (n=9) treated with 120mM NaCl for 30s (pink bar). Traces show elevations in cytosolic Ca\(^{2+}\), measured as increases in Oregon Green BAPTA fluorescence compared to that of Texas Red. B Overlay of multiple *cax1-kd91* cells (n=11) treated with 120mM NaCl for 30s (pink bar). C Mean traces of *cw15* (blue) and *cax1-kd91* (red) cells treated with 120mM NaCl. D Percentage of cells of *cw15* and *cax1-kd91* which showed a NaCl-induced Ca\(^{2+}\) elevation. E Duration, in s, of the Ca\(^{2+}\) increase in response to 120mM NaCl stress in *cw15* and *cax1-kd91*. F Time of the first Ca\(^{2+}\) elevation in response to 120mM NaCl stress in *cw15* and *cax1-kd91*, measured as time after commencement of imaging (0s).
Figure 5.3: *C. reinhardtii* cw15 and cax1-kd91 cells

A *Cw15* cells imaged at 100X magnification, showing flagella at the apex. B *Cax1-kd91* cells imaged at 100X magnification, showing no flagella, which was true of the results from cells presented in Figure 5.1. (Images representative of 10 images for each *cw15* and *cax1-kd91*).
Reduced cytosolic acidification in \textit{cax1-kd91} after acid shock:

It was interesting to explore the effect of acid shock on the response of \textit{CAX} knockdowns compared with \textit{cw15} for two reasons. Firstly, as addition of benzoate has been shown to elicit \(\text{Ca}^{2+}\) spiking associated with deflagellation in \textit{C. reinhardtii} (Wheeler et al. 2008). Secondly, because plant CAXs may play a role in pH regulation, as they are themselves regulated by pH (Pittman et al. 2005) and undergo joint regulation with the V-ATPase at the vacuole membrane (Barkla et al. 2008). Oregon Green BAPTA Dextran could not be used in order to image \(\text{Ca}^{2+}\) transients in \textit{C. reinhardtii} in response to acid shock, as it is responsive to low pH (pH 5.0) (Molecular Probes Handbook). However, pH imaging using the pH-responsive BCECF was used in order to gauge whether lack of CAX activity might lead to differences in the pH response of \textit{C. reinhardtii} cells to acid shock with 10mM and 60mM benzoate (pH 6.0) (Figure 5.4).

A single addition of 10mM benzoate to \textit{cw15}, \textit{cax1-kd91} and \textit{cax2-kd1} cells caused a temporary decrease in pH as indicated by a reduction in the fluorescence of BCECF at 495nm compared with at 440nm (Figure 5.4 A, C, E). When the individual pH traces for each cell line were averaged, \textit{cw15} and \textit{cax1-kd91} showed a similar acidification, while \textit{cax2-kd1} showed a slightly lower decrease in the fluorescence of BCECF at 495nm compared to at 440nm (Figure 5.4 G), although this difference might be partially explained by a small number of cells showing a stronger acidification in \textit{cw15} and \textit{cax1-kd91} (Figure 5.4 A, C). A more noticeable difference in pH was observed between \textit{cw15} and \textit{cax1-kd91} cells treated with 60mM benzoate (Figure 5.4 B, D). When the traces for each cell were averaged, although showing a similar pattern of rapid acidification in response to the acid shock, \textit{cax1-kd91} cells showed a 0.1, or 25\%, reduction in acidification compared to \textit{cw15} (Figure 5.4 F).
Figure 5.4: Response of *C. reinhardtii* CAX knockdowns to acid shock

(Figure legend presented overleaf).
Figure 5.4 (legend):

**A** Change in pH of multiple *cw15* cells (n=17) treated with 10mM pH6.0 sodium benzoate, applied at 100s. Change in pH measured using BCECF-AM dye, based on the ratio of emission wavelengths generated after excitation at 488nm and 458nm. **B** Change in pH of multiple *cw15* cells (n=22) treated with 60mM pH6.0 sodium benzoate. **C** Change in pH of multiple *cax1-kd91* cells (n=15) treated with 10mM pH6.0 sodium benzoate. **D** Change in pH of multiple *cax1-kd91* cells (n=18) treated with 60mM pH6.0 sodium benzoate. **E** Change in pH of multiple *cax2-kd1* cells (n=17) treated with 10mM pH6.0 sodium benzoate. **F** Mean change in pH of *cw15* (blue) and *cax1-kd91* (red) cells in response to 60mM benzoate. Standard error of the mean is shown for each time point. **G** Mean change in pH of *cw15* (blue), *cax1-kd91* (red) and *cax2-kd1* (green) in response to 10mM benzoate. Standard error of the mean is shown for each time point.
Discussion:

The role of CAX in Ca\textsuperscript{2+} signalling in *C. reinhardtii*

The evidence presented here suggests that knockdown of *CAX1* leads to an altered Ca\textsuperscript{2+} signalling response to NaCl stress in *C. reinhardtii*. However, it must be noted that only one *CAX1* knockdown in the cell wall-less *cw15* background has been imaged thus far due to difficulties in measuring reproducible Ca\textsuperscript{2+} elevations in the walled 11/32C background. The results are therefore interesting, and will be discussed tentatively, but for now represent initial findings which have yet to be reproduced in another *CAX1* knockdown. It is of particular importance to measure cytosolic Ca\textsuperscript{2+} signalling in other CAX knockdowns considering that initially, *cax1*-kd91 appeared to show completely reduced Ca\textsuperscript{2+} signalling in response to NaCl stress, but later showed a present, yet still much reduced response compared to *cw15*. At this point, the reasons for this apparent difference are unclear. It is possible that an absence of flagella in *cax1*-kd91 might have rendered cells further unable to respond to NaCl stress in the first round of experiments. Alternatively, it might be that the amiRNA knockdown of *CAX1* in *cax1*-kd91 has lost efficacy over time. Notwithstanding the differences between the two experiments, however, the results as a whole suggest that *cax1*-kd91 shows a much reduced capacity to respond to 120mM NaCl stress, as well as an apparent inability to properly maintain a resting cytosolic Ca\textsuperscript{2+} concentration.

It was predicted that knockdown of *CAX1* would lead to a reduced capacity of *cax1*-kd91 cells to efflux Ca\textsuperscript{2+} following a stimulus-induced cytosolic Ca\textsuperscript{2+} elevation, resulting in an elongated Ca\textsuperscript{2+} increase. This was not the case, however, with several *cax1*-kd91 cells in one experiment showing a Ca\textsuperscript{2+} elevation similar to wild type in response to 120mM NaCl. It is possible that, in *cw15* cells, CAX1 and CAX2 contribute to reducing Ca\textsuperscript{2+} after NaCl-induced Ca\textsuperscript{2+} spiking, but in *CAX1* knockdowns redundancy in function means that other Ca\textsuperscript{2+} transporters, such as Ca\textsuperscript{2+}-ATPases, might rescue the disruption in CAX activity. It might also be that Ca\textsuperscript{2+}-ATPases are primarily responsible for
reducing Ca\(^{2+}\) to resting concentration after a NaCl-induced spiking event. This would contradict evidence from modelling studies in yeast, which suggest that, in response to external Ca\(^{2+}\) stress, the native yeast Ca\(^{2+}\)/H\(^+\) antiporter Vcx1 can more effectively reduce cytosolic Ca\(^{2+}\) back to resting concentration than the Ca\(^{2+}\) pump Pmc1 (Cui et al 2009). However, in rice plants treated with NaCl stress, the expression of various OsCAX isoforms was largely unaffected by treatment with 150mM NaCl in either a salt tolerant or salt sensitive variety, whereas expression of OsACA4 dramatically increased in the salt tolerant rice strain, and OsACA6 expression dramatically increased in the salt sensitive variety (Yamada et al. 2014). Interestingly, the expression of OsCAX3 and OsCAX4 did slightly increase in both the salt tolerant and salt sensitive rice strain in response to 150mM NaCl treatment (Yamada et al. 2014). However, the only real difference between the rice strains in terms of CAX expression was a reduced expression of OsCAX1a in the salt tolerant rice variety compared with the salt sensitive variety (Yamada et al. 2014). Along with evidence that ACA4 can be upregulated in cax1 mutants (Cheng et al. 2003), while CAX1 expression in C. reinhardtii appears to decrease at NaCl concentrations of 100 and 200mM compared to control (Pittman et al. 2009) there is a possibility that CAX1 and CAX2 function is not entirely necessary in order for Ca\(^{2+}\) extrusion from the cytosol in response to NaCl-induced Ca\(^{2+}\) elevations.

A more likely case for the function of CAX1 in the Ca\(^{2+}\) signalling of NaCl stress in C. reinhardtii, therefore, might be that CAX1 is responsible for loading intracellular stores prior to stimulus induced Ca\(^{2+}\) release via Ca\(^{2+}\) channels residing in the membranes of organelles. The apparent lack of ability of many cax1-kd91 cells to maintain a proper resting cytosolic Ca\(^{2+}\) concentration, with cells instead exhibiting random Ca\(^{2+}\) oscillations, supports this hypothesis. The much reduced capacity of cax1-kd91 cells to elevate cytosolic Ca\(^{2+}\) concentration in response to NaCl compared to cw15 cells might also be indicative of disrupted intracellular loading of Ca\(^{2+}\), as the results presented in Chapter 4 suggest that the main Ca\(^{2+}\) elevation is derived from intracellular stores following a Ca\(^{2+}\)
influx from external stores. The reduced capacity of *Arabidopsis cax1/cax3* mutants to properly sequester ions into a central vacuole (Punshon et al. 2012), contrasting with the excessive Ca\(^{2+}\) sequestration displayed by tobacco CAX1 overexpressors (Hirschi 1999), also supports the case for an especially important function of CAX to be in properly maintaining a balanced intracellular store of cations, including Ca\(^{2+}\).

A reduced activity of CAX might also explain the difference between *cw15* and *cax1*-kd91 cells in response to acid shock, with the CAX1 knockdown showing a reduced acidification compared to *cw15* at 60mM benzoate. This could perhaps be confirmed by analysing the Ca\(^{2+}\) signalling phenotype of *cax1-kd91* using Fluo-4-dextran, which is less pH sensitive than Oregon Green BAPTA. It would be interesting in the future to measure how populations of *cax1-kd91* cells fare in response to low pH treatment, considering that *Arabidopsis cax3* mutants show an increased sensitivity to low pH (Zhao et al. 2008). The low pH sensitive phenotype of *cax3* plants was attributed to a reduced activity of plasma membrane H\(^+\)-ATPases (Zhao et al. 2008), while vacuolar H\(^+\)-ATPase activity was reduced by ~25\% in *cax1, cax2, cax3, cax1/cax2* and *cax2/cax3* *Arabidopsis* mutants (Connorton et al. 2012). H\(^+\) transport across membranes is a major mechanism of pH regulation, and CAX transporters may have a large but transient effect on cytosolic acidification due to the transport of 3H\(^+\) for each Ca\(^{2+}\) (Pittman 2012). CAX function in relation to pH maintenance may also not be confined to the cytosol, considering that CAX1 and CAX3 of *Arabidopsis* have been shown to contribute to the regulation of apoplastic pH surrounding guard cells (Cho et al. 2012). It is therefore possible that differential regulation of H\(^+\) channels and translocators, including CAX1 and CAX2, in *cax1-kd91* is responsible for the slightly lower cytosolic acidification observed in response to benzoate.

**Conclusion and perspectives:**

It appears that in the CAX1 knockdown *cax1-kd91*, if not entirely abolished, Ca\(^{2+}\) signalling is altered compared to that observed in *cw15* C. *reinhardtii*. As only one CAX1
knockdown was imaged, however, these results will need to be confirmed in other CAX knockdown lines. Although the phenotypes presented for cax1-kd91 support the observations of Chapter 3, including altered Ca\(^{2+}\) accumulation and sensitivity to CaCl\(_2\) and NaCl stress, confirmation via more CAX1 knockdowns is required to rule out effects due to random mutagenesis via insertion of the CAX1 amiRNA vector during transformation. Attempts were made to image cytosolic Ca\(^{2+}\) transients using the wild type 11/32C background but imaging was difficult as the cell walled C. reinhardtii cells stuck to slides using their flagella and tended to either wobble excessively, or cells would glide away using gliding motility. However, repeated experiments on cax1-kd91 suggest that, rather than being responsible for modulating Ca\(^{2+}\) signals in response to NaCl stress in C. reinhardtii, CAX1 might instead be responsible for correctly loading intracellular Ca\(^{2+}\) stores prior to NaCl induced Ca\(^{2+}\) release, which could explain the lower percentage of cax1-kd91 cells exhibiting a cytosolic increase in Ca\(^{2+}\). It also appears that CAX function might be necessary in order to maintain resting cytosolic Ca\(^{2+}\) concentration in C. reinhardtii, considering the frequent fluctuations in cytosolic Ca\(^{2+}\) observed in cax1-kd91 compared to the relatively flat baseline of cw15 cells. It will be important in the future to measure Ca\(^{2+}\) signalling in other CAX knockdowns in order to confirm the phenotype observed in cax1-kd91. It would also be interesting to gauge whether C. reinhardtii cax1 mutants would show a more pronounced phenotype than CAX1 knockdowns, when CAX1 function is entirely abolished.
Chapter 6 - Final discussion:

The role of CAX1 and CAX2 in C. reinhardtii:

This thesis aimed to further elucidate the role of CAX proteins in both maintaining ion homeostasis and in modulating Ca\(^{2+}\) signals in C. reinhardtii. As localisation of both proteins was unsuccessful, it is difficult yet to speculate as to their precise roles in relation to the phenotypes presented here. The altered accumulation of various metals in several C. reinhardtii CAX1 knockdown lines suggests that CAX1 of C. reinhardtii may be important in maintaining a proper balance of nutrients in C. reinhardtii, while the increased sensitivity of CAX1 knockdowns to Ca\(^{2+}\), Cd\(^{2+}\), and Na\(^{+}\), suggests a role for CAX1 in maintaining ion homeostasis and providing tolerance to metal stress.

Considering, however, that both CAX1 and CAX2 were knocked down in each of the CAX1 and CAX2 knockdown lines, it is difficult to understand the precise role that CAX1 and CAX2 might play. This relationship is especially difficult to understand before future experiments can be performed, considering the opposite phenotypes in relation to ion stress presented by the CAX1 and CAX2 knockdowns. However, it certainly appears that CAX1 and CAX2 affect the response to a similar range of stresses as do plant CAXs, while perturbing CAX activity in both plants and C. reinhardtii leads to an altered ability for cells to properly sequester nutrients. It will be important in the future to properly investigate the roles of, and possible interaction between, CAX1 and CAX2, and especially the effect of knocking down these proteins on the activity of other transporters, such as H\(^{+}\)-ATPases, which might partially explain the altered nutrient accumulation in CAX1 knockdowns. It will also be interesting to explore the nature of the dual knockdown of CAX1 and CAX2, and further investigate the relationship between these proteins during the response of C. reinhardtii to ion stresses. The imminent arrival of the Carnegie Institute for Science C. reinhardtii mutant library might yield some useful cax1 and cax2 knockout lines which could be used to investigate the precise role of each protein.
The role of CAX1 in shaping Ca$^{2+}$ signals in *C. reinhardtii*:

Due to the nature of the Ca$^{2+}$ signalling phenotype observed in *cax1-kd91*, it is difficult to speculate as to the precise nature of the role of CAX in modulating Ca$^{2+}$ signals in *C. reinhardtii*. It was hypothesised that knockdown of CAX1 would lead to a reduced capacity for cells to efflux Ca$^{2+}$ from the cytosol following a stimulus-induced cytosolic Ca$^{2+}$ elevation. This was not the case with *C. reinhardtii cax1-kd91*, however, which either showed an absence of cytosolic Ca$^{2+}$ increases in response to NaCl, or showed a response similar to wild type cells but with less than half of cells responding to the NaCl stimulus. This might be due to some residual CAX activity due to only the partial knockdown of CAX1 and CAX2 as presented in Chapter 3, or due to activity of other Ca$^{2+}$ transporters, such as Ca$^{2+}$ pumps. It might be that, as may tentatively be the case with V-ATPase deficient *Arabidopsis det3* mutants (Allen et al. 2000), reduction in the capacity of cells to properly sequester Ca$^{2+}$ might lead to a disruption in the ability of cells to form cytosolic Ca$^{2+}$ transients.

In support of this, Ca$^{2+}$ imaging experiments revealed that *cax1-kd91* cells were unable to maintain a resting cytosolic Ca$^{2+}$ concentration, with cells instead showing an oscillation in the baseline level of Ca$^{2+}$, regardless of NaCl stress. Although hypothesised to localise to intracellular, vacuole-like acidocalcisomes in *C. reinhardtii* due to vacuolar localisation in transformed yeast cells (Pittman et al. 2009), localisation of CAX1 and CAX2 has not yet been demonstrated *in alga*. However, it is likely that CAX1 function is necessary in order to correctly sequester Ca$^{2+}$ into intracellular compartments prior to stimulus-induced Ca$^{2+}$ release. This seems especially likely considering the wave-like pattern of cytosolic Ca$^{2+}$ observed under CaCl$_2$ and NaCl stress presented in Chapter 4, which suggests intracellular Ca$^{2+}$ release from stores follows an apically-generated Ca$^{2+}$ increase requiring an external source of Ca$^{2+}$. Additionally, the evidence presented in Chapter 3 suggests that knockdown of CAX1 might lead to a disruption in the capacity of cells to properly sequester Ca$^{2+}$. The difference in the acidification profile of *cax1-kd91*
compared with *cw15* does suggest that a lack of CAX function might lead to a disturbance in the pH regulation of cells during a known Ca\(^{2+}\) spike-eliciting stimulus (Wheeler et al. 2008). Further probing of this phenotype will be necessary in order to gauge whether this is due to a reduction in Ca\(^{2+}\)/H\(^+\) antiport by CAXs, or whether knockdown of *CAX1* has a downstream effect on the activity of other H\(^+\) transporters.

Rather than having a direct role in modulating Ca\(^{2+}\) signals in response to NaCl stress in *C. reinhardtii*, CAX1 might instead be involved in indirect regulation of other NaCl responsive elements. This seems especially likely considering that while in *CAX1* and *CAX2* knockdowns both genes show a similar extent of downregulation, Na\(^+\) tolerance is lesser in *cax1-kd91*, *cax1-kd19* and *cax1-kd21*, but greater in *cax2-kd1*. If it were simply that a lack of CAX activity would lead to differential Na\(^+\) transport via CAX transporters, then it might have been expected that both *CAX1* and *CAX2* knockdowns would present a similar phenotype. The opposing phenotypes, therefore, might be explained by the knocking down of *CAX1* and *CAX2* having important, yet differing, downstream effects on the activity of other genes and proteins, including transcription factors and protein kinases. The evidence from *Arabidopsis* that CAX1 is involved in regulating salt-responsive proteins including SOS1 and SOS2, as part of a salt-sensitive response pathway (Ji et al. 2013), adds weight to this observation, as does evidence that cold-responsive *CBF/DREB1* transcription factors are differentially regulated in *cax1* *Arabidopsis* mutants (Catalá et al. 2003). The observed reduction in *NCX1* expression in *cax2-kd1* presented in Chapter 3 also suggests that the activity of CAXs in *C. reinhardtii* has an effect on the activity of at least one putative Na\(^+\) transporter, which is more interestingly a putative Na\(^+\)/Ca\(^{2+}\) exchanger.
The putative localisation of *C. reinhardtii* CAX1 and CAX2:

The increase in Ca content observed in *cax1-kd91* in both control media and at 10mM CaCl₂ is perhaps unexpected, considering that this increased accumulation has otherwise been typical of CAX overexpressors (Hirschi et al. 2000, Mei et al. 2007, Mei et al. 2009) due to increased vacuolar Ca²⁺ sequestration. *Chlamydomonas* CAX1 localises to the vacuole when expressed in yeast cells (Pittman et al. 2009) as does CAX2 (Rachel Webster, previous data), therefore it was hypothesised that CAX1 and CAX2 would localise to an acidic intracellular compartment in *C. reinhardtii*, such as an acidocalcisome, in order to sequester high levels of Ca²⁺.

It is not impossible that either CAX1 or CAX2 encodes a plasma membrane transporter in *Chlamydomonas*, considering that when transformed into onion epidermal cells and *Arabidopsis*, CAX1 of soybean localised to the plasma membrane (Luo et al. 2005). Transgenic *Arabidopsis* plants were also shown to accumulate less Na, Li and K than wild type plants, which is similar to the effect of knocking down CAX1 in *C. reinhardtii*, in which *cax1-kd91* and *cax1-kd21* showed reduced K content at 10mM CaCl₂. A lack of Ca²⁺ efflux at the plasma membrane might also explain the increased Ca²⁺ content of *cax1-kd91*, not necessarily directly due to perturbed CAX activity but indirectly due to its effect on other Ca²⁺ transporters, such as NCX1. Furthermore, the increase in Ca content observed in *cax1-kd91* might also be explained by the diversion of Ca²⁺ sequestration toward another internal compartment, such as the endoplasmic reticulum, via different Ca²⁺ transporters in *C. reinhardtii* such as Ca²⁺-ATPases. Certainly, knockdown of *NCX1* was observed in *cax2-kd1* (Figure 3.1 D), while in *Arabidopsis cax1* mutants the Ca²⁺-ATPase gene *ACA4* was overexpressed (Cheng et al. 2003), therefore altered activity of a global Ca²⁺ efflux network in *C. reinhardtii* is possible.

An analysis of the various metal transporters of *C. reinhardtii* along with predicted targets for each transporter also suggests that CAX1 localises to a vacuolar-type organelle, while CAX2 is also likely to localise to an intracellular organelle, which was
defined as either the chloroplast or the mitochondria (Hanikenne et al. 2005). Ca\(^{2+}\)/H\(^{+}\) antiport activity has been previously observed at the thylakoid membrane of peas and was shown to be dependent on a pH gradient, which suggests that direct antiport of Ca\(^{2+}\) for H\(^{+}\) was occurring in response to light and ATP hydrolysis (Ettinger et al. 1999). Despite a lack of identification of a thylakoid-specific CAX isoform, it is possible that either CAX1 or CAX2 of C. reinhardtii might localise to the chloroplast. However, the overwhelming majority of CAX proteins localise to the vacuole of plants, as well as transformed yeast cells (Manohar et al. 2011). Recently, CAXs from the T. gondii and P. berghii parasites were shown to localise to small vesicles scattered throughout the cytosol (such as acidocalcisomes (Figure 6.1)) and other vesicles similar to vacuole-like compartments (Guttery et al. 2013). It is likely that localisation of CAX1 and CAX2 occurs at a similar intracellular location in C. reinhardtii, especially considering that phylogenetic analysis shows algal CAXs group very closely with protozoan CAXs (Pittman et al. 2009). In order to better understand the role of CAX transporters in C. reinhardtii the next important step is to properly localise CAX1 and CAX2.
Figure 6.1: Acidocalcisome stain in *cw15* *C. reinhardtii*

**A** *cw15* cell imaged using epifluorescence microscopy at 100X magnification after treatment with LysoTracker Red for 30min. Blue dots indicate acidic compartments scattered throughout the *C. reinhardtii* cytosol. Chloroplast autofluorescence is red.  

**B** *cw15* cell imaged at 100X magnification after treatment with BCECF/AM for 30min. Green dots indicate acidic compartments scattered throughout the *C. reinhardtii* cytosol. Chloroplast autofluorescence is red.
The flagella hypothesis:

A particularly interesting feature of the response of *C. reinhardtii* cells to NaCl stress was the lack of Ca\(^{2+}\) signal observed in *cax1-kd91* cells lacking flagella, compared to the reduced, but still present, Ca\(^{2+}\) signalling response in flagellated cells. Combined with evidence that NaCl and CaCl\(_2\) stress causes *C. reinhardtii* cells to exhibit an initial apically-generated Ca\(^{2+}\) increase, which is then followed by what appears likely to be an intracellular Ca\(^{2+}\) store-derived Ca\(^{2+}\) wave through the cytosol, it is tempting to speculate that osmotic stress is detected by the flagella, and then relayed throughout the *C. reinhardtii* cytosol. I have therefore devised a “flagella hypothesis” to attempt to explain how *C. reinhardtii* cells might respond to environmental stimuli, such as NaCl stress (Figure 6.2).

The *adf1* mutant, proposed to be defective in a component of a *C. reinhardtii* Ca\(^{2+}\) influx channel (Finst et al. 1998) present in the cell membrane at the flagella transition zone (Quarmby and Hartzell 1994), provides evidence for this, considering that *adf1* mutants show no Ca\(^{2+}\) spiking at the apex in response to benzoate addition, as well as an inability to shed flagella in response to the acid shock (Wheeler et al. 2008). Other potential Ca\(^{2+}\) channels present at the cell membrane include various homologues of the recently described osmosensing OSCA1 of *Arabidopsis* which have been suggested in the *C. reinhardtii* genome (Yuan et al. 2014). Furthermore, the abundant presence of mechanosensitive TRP channels in *C. reinhardtii*, several of which have been localised to the flagella and linked to Ca\(^{2+}\) dependent responses (Huang et al. 2007, Fujii et al. 2011, Collingridge et al. 2013), suggests there are several Ca\(^{2+}\) channels residing at the flagella region, or at the cell membrane, which are of interest to study in terms of generating Ca\(^{2+}\) transients in response to external stresses. It would be interesting in the future to identify NaCl-stimulated Ca\(^{2+}\) channels in *C. reinhardtii* and investigate whether reduction in function would have an effect on the NaCl-induced Ca\(^{2+}\) elevations presented in Chapter 4. Based on the observation that external Ca\(^{2+}\) is required in order to generate cytosolic...
Ca$^{2+}$ transients, as well as evidence of a Ca$^{2+}$ wave in response to CaCl$_2$ and NaCl stress, we propose that this initial Ca$^{2+}$ influx then triggers a larger Ca$^{2+}$ increase from intracellular Ca$^{2+}$ stores, which then spreads rapidly throughout the cell and activates a number of downstream responses, potentially including deflagellation. Considering that Ca$^{2+}$ treatment alone can cause deflagellation (Sanders and Salisbury 1994, Wheeler et al. 2008), it will be interesting to explore whether deflagellation is a general response of *C. reinhardtii* in response to sudden environmental stress.
NaCl stress triggers receptor proteins at either the cell membrane or the flagella, which causes influx of Ca\(^{2+}\) via a Ca\(^{2+}\) permeable channel (such as a transient receptor potential channel (TRP) or reduced hyperosmolality induced [Ca\(_i\)] increase channels (OSCA)) activated by either Ca\(^{2+}\), pH, Na\(^{+}\), or stretching due to the osmotic shock. The initial Ca\(^{2+}\) increase triggers release of Ca\(^{2+}\) from intracellular stores (small green circles) (loaded by calcium/proton exchangers (CAX)) via Ca\(^{2+}\) channels such as inositol-4,5-trisphosphate receptor (IP\(_3\)R)-like channels. The Ca\(^{2+}\) elevation spreads as a wave throughout the C. reinhardtii cytosol, and forms part of a signal transduction mechanism which leads to activation of stress responses such as differential gene expression, altered activation of transporters such as an Na\(^{+}/H^+\) exchanger (NHX) or H\(^+-\)ATPases, and potentially deflagellation.

**Figure 6.2: A proposed Ca\(^{2+}\) signalling mechanism for C. reinhardtii cells in response to NaCl stress**
Future work:

There remains a great deal to investigate in order to precisely gauge the role CAX proteins play in both maintaining ion homeostasis and modulating Ca\(^{2+}\) signals in *C. reinhardtii*. It will be of particular importance to investigate the relationship between CAX1 and CAX2, considering their dual knockdown after amiRNA but apparently opposing phenotypes in response to CaCl\(_2\) and NaCl stress. Localisation of both CAX1 and CAX2 will also help greatly in explaining their physiological role in *C. reinhardtii* cells. Further investigation into the role of NCX1 might also aid explaining how *C. reinhardtii* responds to environmental stresses, especially since *NCX1* expression appears to be relatively highly inducible after growth of cells in 10mM CaCl\(_2\). Finally, it will be interesting to further explore the mechanisms of Ca\(^{2+}\) signalling in *C. reinhardtii* in response to NaCl stress, as well as other potential Ca\(^{2+}\) signal-inducing environmental stimuli. The following is a list of experiments which would be pertinent to perform in the near future:

- Characterisation of CAX1 and CAX2. CAX1 and CAX2 should be localised in *C. reinhardtii* cells, therefore gDNA and cDNA should be ligated into expression vectors with fluorescent tags, such as mCherry, mTurquoise and mVenus, and overexpressed in *C. reinhardtii* before imaging using deconvolution microscopy. Further phenotypic analysis of *CAX1* and *CAX2* knockdowns and overexpressors, as well as analysis of *cax1* and *cax2* mutants, including investigation into the possible co-regulation of CAX1 and CAX2, might help to explain the role of CAX proteins in *C. reinhardtii*.

- Characterisation of NCX1. *NCX1* should be knocked down using amiRNA and analysed in a manner similar to the *CAX1* and *CAX2* knockdowns presented in this Thesis, in order to gauge its relative importance in maintaining ion homeostasis and modulating Ca\(^{2+}\) signalling, particularly in regard to NaCl stress.

- Ca\(^{2+}\) and pH imaging. Imaging experiments should be performed on further *CAX1* and *CAX2* knockdowns, in order to confirm whether disruption in CAX activity in *C. reinhardtii*...
*reinhardtii* causes a Ca$^{2+}$ signalling-deficient phenotype, as well as altered cytosolic pH regulation. The role of flagella in receiving stimuli such as NaCl could be explored by imaging Ca$^{2+}$ in *C. reinhardtii* cells with and without flagella, as well as imaging the flagella themselves. It would also be interesting in the future to identify possible NaCl-sensitive Ca$^{2+}$ permeable channels at the cell apex, or possibly at the flagella, which allow Ca$^{2+}$ influx in response to NaCl stress. The requirement of an intracellular store of Ca$^{2+}$ could be investigated by using antagonists of known ligand-gated Ca$^{2+}$-channels, such as IP$_3$R-like channels and CNGCs, during Ca$^{2+}$ imaging experiments. Finally, the activation of downstream responses such as gene expression of salt-specific genetic motifs could also be investigated, using knowledge of *Arabidopsis* salt-sensitive genetic elements, as well as methods such as RNA sequencing (RNA-Seq).

**Conclusion:**

The results presented in this Thesis show, for the first time, that *C. reinhardtii* responds to environmentally relevant stimuli with rapid, transient Ca$^{2+}$ signalling through the cytosol which is similar to that observed in plant and animal cells. The mechanisms shaping these signals across all organisms have yet to be fully defined, therefore further study into *C. reinhardtii* Ca$^{2+}$ signalling could provide interesting insights into the evolution of animal and plant stress signalling responses, including the different transporters responsible for generating and modulating Ca$^{2+}$ transients. The future availability of the *C. reinhardtii* mutant library will allow further investigation into CAX1 and CAX2, as hopefully cax1 and cax2 mutants will provide a more detailed explanation of the relative role of each transporter when function is completely abolished, rather than knocked down. However, the results presented here suggest an important role for CAX1 in maintaining a balance of metal ions, especially Ca$^{2+}$, in *C. reinhardtii*, as well as involvement of both CAX1 and CAX2 in modulating *C. reinhardtii* responses to various ionic stresses.
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